Epigenetic regulation of normal and malignant hematopoiesis

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EPIGENETIC REGULATION OF NORMAL AND MALIGNANT HEMATOPOIESIS

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Chapter 1

Introduction and Thesis Outline

INTRODUCTION

Hematopoiesis; the formation of blood

In our body, billions of red blood cells, white blood cells and platelets are produced daily. This process is essential, since most blood cells are short-lived. In addition, increased cell production may be temporarily needed, for example in case of infection or excessive bleeding. By the enduring activity of hematopoietic stem cells (HSCs), mature blood cells are continuously replenished and HSCs can respond upon demand to produce more cells. In this way, the total number of cells in the peripheral circulation remains more or less constant (homeostasis).

HSCs reside in the bone marrow, which is the spongy, red substance located in the innermost part of bones such as the pelvis, vertebrae, ribs, sternum, skull, and limbs. HSCs require spatial and temporal input from this microenvironment or 'niche', for maturation or self-renewal. Specific cell types in the niche, such as vascular endothelial cells, osteoblasts, adipocytes, mesenchymal stem cells and neurons provide (gradients of) soluble molecules as well as (gradients of) physical factors such as shear stress, oxygen tension and temperature [1]. These external signals, together with intrinsic mechanisms, induce intracellular signaling cascades that determine the behavior of HSCs.

HSCs are typically depicted at the top of a hierarchical 'lineage tree', each branch point of which indicates a restriction in proliferation and developmental potential. The self-renewal ability of HSCs maintains the stem cell pool while their multipotency allows for formation of all mature blood cell types through multiple differentiation steps. Although long-term (LT) HSCs, short-term (ST) HSCs and multipotent progenitors (MPPs) are all multipotent, they differ substantially in their self-renewal capacity. While LT-HSCs possess very extensive self-renewal, they progressively lose their self-renewal potential upon entrance into the differentiation pathway to form ST-HSCs and MPPs. Multipotency is then lost upon either lymphoid or myeloid lineage commitment (Figure 1). Common myeloid progenitors (CMPs) can further differentiate into either granulocyte-macrophage progenitors (GMPs) which will produce mature leukocytes (white blood cells) such as granulocytes (eosinophils, neurophils, basophils) and macrophages, or megakaryocyte-erythroid progenitors (MEPs) which will produce erythrocytes (red blood cells) and thrombocytes (platelets) [2]. Common lymphoid progenitors (CLP) will differentiate into mature B- and T-lymphocytes and NK-cells (leukocytes) [3].

Hematopoietic stem cells; their hallmark properties

The nuclear bombing of Hiroshima and Nagasaki in 1945 led to the observation that a prolonged period of radiation compromised the hematopoietic system. People in the affected

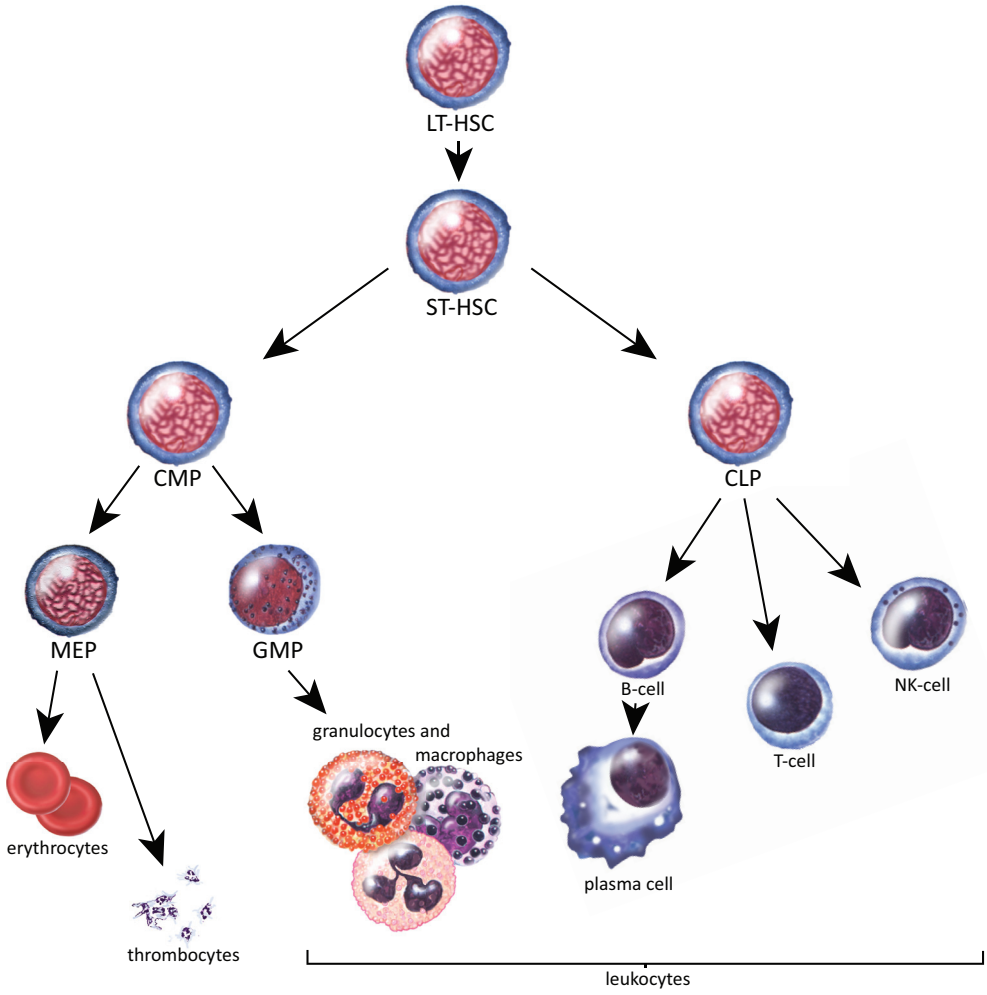

Figure 1: hematopoietic differentiation tree. Abbreviations used: long-term hematopoietic stem cell (LT-HSC), short-term hematopoietic stem cell (ST-HSC), common myeloid progenitor (CMP), common lymphoid progenitor (CLP), megakaryocyte-erythroid progenitor (MEP), granulocyte-macrophage progenitor (GMP). *Modified from © 2008 Terese Winslow, U. S. Govt.*

area suffered from low blood cell counts and platelets, resulting in bleeding disorders and increased sensitivity towards infections [4-6]. In addition, increased leukemia incidence occurred in persons that were exposed and whose blood counts recovered after the bomb fell. These observations led to multiple studies on radiation syndromes. In 1948, Bloom and Jacobsen showed that rodents died from hematopoietic failure two weeks after low dose whole body x-irradiation [7-8]. Soon thereafter, it was shown that injections of bone marrow

suspensions from healthy donor mice could rescue irradiated mice from hematopoietic failure 9. In 1956, E. D. Thomas performed the first bone marrow transplant in human, treating patients with advanced leukemia [10]. Although unsuccesfull for a large percentage of attempts, in 1975, due to acquired knowledge of the immunesystem and by matching donors and recipients based on HLA types, this was shown to be an effective treatment. He received the Nobel Prize for Medicine in 1990 [11]. Nowadays, more than 50.000 bone marrow transplants are performed annually. However, at the time E.D. Thomas performed transplantations, it was not known which factors were responsible for the reconstitution of the entire blood system. It took another ten years before it was acknowledged that specific primitive hematopoietic cells were responsible for regeneration of the blood system, and not certain soluble blood factors such as hormones [12-16]. Pioneering experiments were performed by Till and McCulloch. They showed that ten days after transplantation of a limited number of bone marrow cells, colonies were formed on the spleen of recipient mice [12-13] . These colonies correlated directly in number with the number of bone marrow cells originally injected, and were of clonal origin, as assessed by induced chromosomal breaks. They proposed the 'stem cell' concept which still holds true; only a very small subpopulation of bone marrow cells possess two remarkable properties 1) they can self-renew and 2) they can from multiple types of mature blood cells, e.g. they are *multipotent*.

Although Till and McCulloch proposed the existence of adult stem cells in the bone marrow in 1961, those stem cells were only identified in the late 1980's after techniques such as multi-color fluorescence activated cell sorting (FACS) were developed [17]. HSCs were phenotypically identified after the use of monoclonal antibodies was implemented; murine HSCs lack cell surface markers that are generally expressed on mature blood cells ("lineage negative"), but are positive for "stem cell antigen-1 (Sca1)" and "c-Kit" [18]. These "LSK cells" comprise approximately 0.05% of the adult mouse bone marrow cells. Nowadays, several additional marker combinations (such as CD34-, Flt3-, CD150+ and EPCR+ [19-22)] have been developed to more precisely identify the most primitive HSCs within the LSK population. These cells are termed "long-term HSCs" since they possess long-term hematopoietic reconstitution capacity, and are as infrequent as 1:15.000 bone marrow cells.

HSC assays, combined with the ability to purify HSCs, have provided increasingly detailed insight into the unique properties of these cells. The only assay that truly defines the quality of HSCs is a reconstitution assay in which the long-term (>16 weeks) presence of donor-derived cells in a reconstituted host is assessed. It measures both the long-term self-renewal and multilineage differentiation capacity of HSCs. Serial transplantation experiments, in which the bone marrow from a previously transplanted and reconstituted mouse is the new HSC source for a newly irradiated mouse, showed that HSCs can outlive their original donor [23-24]. This clearly reflects their excessive self-renewal potential. However, it is still

unclear which key signals are required for self-renewal. This is also the reason why it has proven difficult to maintain HSCs in culture, or to expand them. Attempts have been made with known stem-cell stimulators such as Stem-Cell Factor (cKit ligand), Interleukins -1, -3, -6 and -11 and Flt3-ligand, or with small molecules such as valproic acid [25-26]. However, although some HSC potential can be maintained, their absolute frequency and self-renewal potential is quickly lost upon transfer in culture, since they tend to differentiate spontaneously. Several *in vitro* assays have been developed to measure the frequency and quality of progenitors and HSCs, such as the colony-forming-unit cell (CFU-C) assay and the cobblestone-area forming cell (CAFC) assay [27]. The CFU-C makes use of the capacity of progenitors to rapidly proliferate and generate many differentiated cells when stimulated with growth factors. When cultured in semi-solid media these cells will produce a number of countable colonies, which reflects the frequency of progenitors in the initial culture. In the CAFC assay, hematopoietic cells are grown on a stromal feeder layer without the addition of growth factors. HSCs maintain a specific and easily recognizable way of growing under the stromal cells for at least five weeks, while progenitors can only form these so-called 'cobblestones' for shorter periods [27]. Although these *in vitro* assays have been shown to correlate with *in vivo* activity [28], transplantation assays are still the ultimate model to test for HSC self-renewal and multipotency.

Leukemia and Leukemic Stem Cells; when HSCs turn bad

By precisely balancing self-renewal and differentiation, HSCs continuously provide differentiated progenitors and mature blood cells while simultaneously maintaining a proper HSC pool size throughout life. This balance is crucial, since impaired self-renewal can result in tissue dysfunction while excessive self-renewal can result in blood cancer (leukemia). In addition, the formation of different blood lineages must be tightly controlled. Too many or too few cells of any particular type can result in disease [29-31]. Erythrocytes are responsible for oxygen transport to all tissues. A deficit of erythrocyte production (anemia) can cause symptoms such as fatigue, dizziness, and organ failure. Excess erythrocytes (polycythemia) increases viscosity of the blood, which can result in microvascular complications as a consequence of blood clot formation (thrombosis). Microvascular complications, such as infarcts and embolisms, can also occur as a consequence of excessive platelet production (thrombocytemia or thrombocytosis). However, since the primary function of platelets is to initiate blood clot formation for wound healing, too few platelets (thrombocytopenia) can result in bleeding disorders. Leukocytes have their primary function in the innate and adaptive immune system, and low white blood cell numbers (leukopenia) can therefore result in deficiencies in combatting infections. Excessive production of (immature) white blood cells is a sign of chronic or acute leukemia,which is typically characterized by a rapid growth and accumulation of immature hematopoietic cells in the bone marrow. A wide variety of leukemias exist, usually classified by the predominant pathologic cell types and/

or the clinical course of the disease. The most common major types are Acute Myeloid Leukemia (AML), Acute Lymphoblastic Leukemia (ALL), Lymphomas (e.g. Hodgkin and non-Hodgkin), and Chronic Myeloid Leukemia (CML). Recently, idiopathic thrombocytemia and polycythemia are also considered as subtypes of chronic myeloid blood cancers.

Leukemias are clonal proliferative diseases that arise from HSCs or progenitors, which failed to obey normal regulatory signals that should restrict their self-renewal. It has been found that only a subfraction of cells within the leukemic cell population, termed leukemic stem cells (LSCs), possesses the ability to initiate and sustain leukemia. In patients, these LSCs often express the same immunophenotypical markers ($CD34^+$,$CD38^-$) as normal human HSCs [32-34]. Although it is still under debate whether LSCs truly originate from normal HSCs, it is clear that the leukemic cell population is heterogeneous and comprised of cells that differ in their differentiation and self-renewal capacities [35]. This suggests that the leukemic cell population is composed of a hierarchy that resembles that of the normal HSC compartment. Nowadays, therapies focus on eliminating those critical LSCs that propagate the leukemia instead of the bulk tumor cell population. Therefore, it is highly important to identify the cancer-specific pathways that initiate malignant transformation and that operate in LSCs.

The observation of the age distribution of cancer has been fundamental to the origin of the multi-stage theory of cancer [36]. This theory implies that most cancers arise from the accumulation of multiple mutations, while the probability of acquiring a sufficient number of detrimental mutations increases with time [37]. Tumors were long thought to evolve in a linear fashion, by the sequential acquisition of mutations in tumor suppressor genes or oncogenes [36, 38-39]. Although it is apparent that mutations in genes that provide a selective growth advantage to cells is a hallmark of tumor development, recent studies suggest that not all cells in a cancer are linearly related. After a first initial mutation, cells can branch off due to different secondary mutations, and cells within these two groups can again acquire mutations that makes them distinct from the cells where they originate from. This process can result in a heterogeneous cancer, consisting of cells belonging to different 'clones' with different properties [40-41]. Cancer, including leukemias and lymphomas, has long been viewed as a purely genetic disease. Many patients with leukemia carry an abnormal karyotype as a consequence of chromosomal translocations. These translocations cause in-frame fusion of two otherwise separated genes (e.g. t(15;17) PML-RARa, t(8;21) AML1-ETO, t(9;22) BCR-ABL). The resulting fusion protein often disturbs proper regulation of proliferation, differentiation, growth factor signaling, senescence or apoptosis. However, about a decade ago it became apparent that cancer is also associated with profound epigenetic changes [42-44]. Tumor cells are associated with genome-wide DNA hypomethylation, gene specific hypermethylation, as well as aberrant histone modifications [45-47]. Whereas DNA hypomethylation results in chromosomal instability, both promoter hypermethylation and

specific histone modifications represents a mechanism to cause aberrant activity of genes involved in cell-cycle regulation.

While in normal hematopoiesis the balance between self-renewal and differentiation is tightly controlled, this balance is clearly lost in leukemic cells. Specific pathways that regulate normal HSC function often are the same pathways that are deregulated in leukemias. Therefore, to fully understand which sequence of events in HSCs can cause leukemic transformation, it is highly important to determine the fundamental mechanisms of HSCs. Regulation of the chromatin structure of HSCs, establishes, maintains, and/or propagates their gene expression programs and thus drives their behavior. Proper regulation of these epigenetic processes is therefore essential for balancing HSC self-renewal and differentiation and can prevent from leukemic transformation.

Epigenetic regulation of HSCs: keeping DNA in shape

Although adult stem cells and their differentiated progeny contain identical genetic information, their gene expression patterns, and consequently their functional behavior, differ substantially. This is accomplished by differential accessibility of the DNA for the transcriptional machinery, caused by epigenetic modifications. DNA is not found as a naked molecule in the nucleus but is wrapped around nucleosomes composed of histone octamers, which are the building blocks of chromatin. The manner in which DNA is packed around these nucleosomes can be altered, by placing chemical modifications or 'epigenetic marks' on histone tails or on the DNA itself. These marks can be heritable during mitotic divisions but are reversible as well, by the activity of specific enzymes that can remove these modifications from the DNA or histones.

DNA methylation

The process of DNA methylation involves the addition of methyl groups to cytosine residues in CpG dinucleotides. Most DNA methylation occurs in these so-called CpG islands, which are regions in the genome where the frequency of C- and G-nucleotides that occur next to each other in sequence is high. In mammalian genomes, CpG island can be 300-3000 base pairs in length and most occur in close proximity to promoters of genes [48]. Methylation of cytosines is executed by the action of the DNA-methyl-transferase (DNMT) family [49]. DNMT3a and DNMT3b are de novo methyltransferases that actively start DNA methylation of unmethylated cytosines on genomic loci that need to be silenced for example during differentiation. DNMT1 is thought to be the maintenance methyltransferase, responsible for copying DNA methylation to daughter strands during DNA replication. Until recently, DNA methylation was long thought as being relatively stable and irreversible and DNA demethylation was considered to only passively occur during cell division. However, it is now clear that TET protein family members can actively convert methylated cytosines into

5-hydroxymethylcytosines (5hmC) [50-52]. 5hmC can act as a substrate for further modications (formylcytosine, carbonylcytosine, uracil) after which it will be ultimately replaced with an unmethylated cytosine without the need for DNA replication.

Besides important during the process of cellular differentiation, DNA methylation is also associated with a number of key processes including genomic imprinting, X-chromosome inactivation, and suppression of repetitive elements. Alterations of DNA methylation have been recognized as an important component of cancer development. Cancer cells are associated with genome-wide DNA hypomethylation, while certain genes show specific hypermethylation. In addition, recent genomic studies have identified recurrent somatic mutations in TET and DNMT family members in patients with hematopoietic malignancies [53].

Histone modifications

Histone tails that protrude from the histone octamer are subject to several types of modifications, including methylation, acetylation, phosphorylation, ubiquitination and sumoylation. Histone modifications serve to either loosen or compact the chromatin, and thereby influence the accessibility of the DNA. Histone modifications can also serve to recruit other proteins such as transcription factors, DNA repair and replication factors, and additional chromatin remodelers, which contribute to repression or promotion of transcription [54]. Locus-specific combinatorial patterns of these modifications define the chromatin state which is also referred to as ''the histone code" [55-56]. Some general histone code 'rules' are often observed and applied. For example, histone lysine acetylation (for example on H3K9) is generally associated with open chromatin and active transcription of genes. The overall level of histone acetylation is dictated by opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) enzymes, which add or remove acetyl groups on lysine residues, respectively. Methylation of histone tails can occur at both lysine and arginine residues and is also mediated by two antagonizing groups of enzymes; histone methyl transferases (HMTs), which can actively methylate residues, and histone demethylases (HDMs), which can erase these marks. The addition of up to three methyl groups at individual lysines produces a total of four methyl states: unmethylated, mono, di-, and tri-methylated. According to the specific amino acid residue where these methylation marks occur, they are either associated with active expression of genes (for example H3K4me3, executed by Thrithorax MLL protein) or with gene silencing (for example H3K27me3, executed by Polycomb Ezh2 protein).

In stem cells, lineage-specific genes must remain silenced, yet ready to be activated upon differentiation. To accomplish this, both in embryonic and hematopoietic stem cells, active H3K4me3 and repressive H3K27me3 histone modifications have been shown to coexist on these developmental crucial genes [57-59]. Genomic loci where these specific repressive

and active marks co-occur are termed 'bivalent domains'. Upon differentiation, promoters with bivalent domains are resolved into a monovalent state, either active or repressive. Although certain rules appear to determine whether specific (combinations of) histone modification will result in activation or repression, and thereby specify a certain biological outcome ('acetylation results in gene activation'), prediction of epigenetic regulation is actually not straightforward. Histone combinations have the potential to be very complex; each of the four histones that compose the nucleosome can be simultaneously modified at multiple different sites, with multiple different modifications. In addition, whereas histone acetylation and methylation are widely studied, the effect of other histone modifications such as phosphorylation, sumoylation and ubiquitination are relatively unknown.

Mis-"writing" and mis-"reading" of histone modifications is strongly associated with cancer initiation and progression. MLL rearrangement and deregulation of EZH2 are among the most common mutations in leukemia and other cancers [56, 60-61].

Polycomb group proteins

It is well recognized that epigenetic regulators play a prominent role in maintaining daughter cells of stem cells in an undifferentiated state by faithfully reproducing the epigenome upon self-renewal divisions. However, upon differentiation signals, the chromatin status must be changed in such a way that loci that encode differentiation genes can be exposed to (transcription-) factors that can promote their expression. Epigenetic regulation can therefore be considered a gatekeeper of cellular memory, since it is crucial for establishing, maintaining and propagating transcriptional profiles of the cell.

A specific, well-studied, class of epigenetic modifiers are Polycomb group proteins (PcGs). They induce transcriptional repression of target genes through catalyzation of specific histone modifications [33-34]. PcGs reside in two multimeric protein complexes: Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2). PRC2 consists of three core subunits; Ezh, Suz12 and Eed. The SET-domain containing Ezh proteins catalyze trimethylation of histone 3 lysine 27(H3K27me3), and to a minor extent lysine 9 of histone 3 (H3K9me3). This modification is found to be the initiating step of gene repression. The PRC1 member Cbx can 'read' this epigenetic mark and recruit PRC1 to the particular locus where this mark is established [62-63]. PRC1 consists of four core components Cbx, Ring1, Phc, and Pcgf. Ring1 possesses histone 2A ubiquitination activity [64]. This histone modification is thought to be the final step in stable gene repression since it blocks the movement of RNA polymerase along the DNA [65-66].

Several Polycomb group proteins have shown to be key players in determining cellular fate decisions in HSCs. In HSCs, they repress the transcription of lineage-specific genes. In response to extrinsic or intrinsic signals, PcG proteins can be displaced from these promoters

and be recruited to stem cell specific self-renewal genes to promote differentiation and suppress unlimited proliferation. However, during differentiation into either the myeloid or lymphoid lineage, PcGs not only repress the transcription of self-renewal genes but also of differentiation genes of the alternative lineage.

Polycomb group genes are often deregulated in various types of cancer, including those of the hematopoietic system. Aberrant expression of PcG genes has frequently been detected and mutations in PRC2-encoding genes have recently been suggested as a causative factor in several types of leukemia [60, 67].

OUTLINE OF THIS THESIS

Whereas in *Drosophila* each Polycomb component is encoded by a single gene, the number of genes encoding for PcG proteins has expanded in mammals which resulted in a large combinatorial diversity in PRC1 and PRC2 complex compositions. In this thesis we aim to elucidate the relevance of this diversification and to assess whether Polycomb complexes with different compositions have different biological functions in murine HSCs.

Chapter 1 provides an introduction into the PcG gene family. We describe how Polycomb members have expanded during evolution and comprehensively review recent literature on their importance for normal HSC regulation. After focusing on the fact that PcG are often aberrantly expressed or mutated in hematopoietic malignancies, we will argue that safeguarding the composition of PRC complexes is invaluable for keeping balanced HSCs self-renewal and differentiation divisions.

In **Chapter 2** we describe the role of PcG proteins in HSC aging. Aging of an organism results from tissue dysfunction and degeneration as a consequence of cellular aging. Adult stem cells, such as HSCs, are critically important to replenish the age-associated loss of cells. We review the literature and hypothesize that stem cells, which have a very extensive lifespan, are particularly vulnerable for malignant transformation. We speculate that PcG proteins regulate the balance between aging (by limiting stem cell self-renewal) and the risk of developing cancer (excessive self-renewal).

In **Chapter 3** we studied the biological function of different Cbx-containing PRC1 complexes in HSCs. Analysis of PRC1 complex composition previously showed that each complex contains a single representative of the Cbx family; either Cbx2, Cbx4, Cbx7 or Cbx8. The N-terminal chromodomain of Cbx orthologs can bind H3K9me3 and H3K27me3 14,15, albeit with unequal affinities. Therefore, the Cbx proteins are key components for targeting PRC1

to specific genomic loci to regulate gene expression. We show that Cbx7-containing PRC1 complexes induce self-renewal of HSCs by repressing the expression of progenitor-specific genes. Other Cbx proteins can compete with Cbx7, resulting in Cbx2-, Cbx4-, or Cbx8-containing PRC1 complexes that induce entrance into the differentiation pathway.

In **Chapter 4** we used a novel barcoding approach that allows tracking the progeny of each individually transduced cell in combination with our Cbx7-overexpressing mouse model to identify LSC-derived clones and their behavior. We show that within one leukemia different LSC clones with different self-renewal and differentiation properties can co-exist. Thus, leukemias can be heterogeneous, containing quiescent cells with very delayed latencies, which can be activated upon proliferative stress.

In **Chapter 5** we summarize the findings of this thesis and discuss future perspectives for example how our findings can contribute to the development of novel leukemia therapies.

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Chapter 2

Polycomb-group proteins in hematopoietic stem cell regulation and hematopoietic neoplasms

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2

ABSTRACT

The equilibrium between self-renewal and differentiation of hematopoietic stem cells is regulated by epigenetic mechanisms. In particular, Polycomb-group (PcG) proteins have been shown to be involved in this process by repressing genes involved in cell-cycle regulation and differentiation. PcGs are histone modifiers that reside in two multi-protein complexes: Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2). The existence of multiple orthologs for each Polycomb gene allows the formation of a multitude of distinct PRC1 and PRC2 sub-complexes. Changes in the expression of individual PcG genes are likely to cause perturbations in the composition of the PRC, which affect PRC enzymatic activity and target selectivity. An interesting recent development is that aberrant expression of, and mutations in, PcG genes have been shown to occur in hematopoietic neoplasms, where they display both tumor-suppressor and oncogenic activities. We therefore comprehensively reviewed the latest research on the role of PcG genes in normal and malignant blood cell development. We conclude that future research to elucidate the compositional changes of the PRCs and methods to intervene in PRC assembly will be of great therapeutic relevance to combat hematological malignancies.

INTRODUCTION

The genome of every organism contains a developmental program that results in expression of various collections of genes in various cell types. In most adult tissues and organs, stem cells have been identified that maintain tissue homeostasis, and the quest to identify novel adult stem cell types is ongoing. Adult stem cells have the capacity to self-renew and the ability to differentiate into the mature cell types of the tissue in which they reside. Although adult stem cells and their differentiated progeny contain identical genetic information, their gene expression patterns differ substantially. This is predominantly accomplished by differential accessibility of the DNA for the transcriptional machinery. Along with this, alterations in the epigenetic landscape of the genome affect the transcriptome, the functioning and the behavior of each cell. However, we are only beginning to understand how collections of genes are turned 'on' or 'off' simultaneously, thereby controlling stem cell self-renewal and differentiation.

The hematopoietic system is a particularly well-studied example of a homeostatic tissue. It generates massive numbers of new blood cells during our entire lifespan by means of hematopoietic stem cells (HSCs), which normally reside in the bone marrow. Recent studies of HSCs have advanced our understanding of the epigenetic mechanisms that maintain the balance between self-renewal and differentiation.

One of the main classes of such epigenetic regulators is provided by the Polycomb-group (PcG) proteins. These proteins are histone modifiers, and their activity results in repression of genes involved in cell-cycle regulation and differentiation. Aberrant PcG expression and mutations in PcG genes have been shown to occur frequently in various types of hematopoietic neoplasms. Emerging evidence indicates that leukemic stem cells (LSCs) evolve from hematopoietic stem cells or progenitor cells, which have acquired abnormal expression of genes involved in cell survival and proliferation. This suggests a vital role for PcG-induced epigenetic modifications in malignant transformation.

In our review, we first describe the PcG gene family and how its members have expanded during evolution. We then focus on their molecular function and their importance for normal HSC regulation. Finally, we describe how aberrant Polycomb functions can result in neoplastic transformation of hematopoietic cells.

POLYCOMB-GROUP GENES (PCG GENES)

PcG genes were first discovered in Drosophila melanogaster as key regulators of homeotic (Hox) gene expression [1]. The PcG proteins reside in two main complexes: Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2). In addition, a Pleiohomeotic Repressive Complex (PhoRC) has been identified to be involved in recruitment of PRCs to chromatin [2-4].

PRC2 consists of three core subunits corresponding to Drosophila PcG genes: Enhancer of zeste (E(z)), Suppressor of zeste 12 (Suz12) and Extra sex combs (Esc). In mammals, the PRC2 complex consists of one of the two Ezh orthologs (Ezh1 or Ezh2), Suz12 and one isoform of Eed (Eed1-4) [5-8]. The classical model by which PRC2 induces transcriptional repression involves the activities of Suz12 and Eed, which together contribute to binding the complex to nucleosomes [9] and to the histone methyltransferase (HMTase) activity of SET domain-containing Ezh proteins [5-9]. The capacity of Ezh to transfer methyl groups to lysine 9 and 27 of histone 3 (H3K9me3, H3K27me3) is stimulated by Eed [10-12]. Furthermore, PRC2 can recruit co-factors such as Aebp2, Mtf2, Pcl3, Jarid2 and Phf1, which can modulate its enzymatic activity and recruitment to target genes [13-19]. The well-documented trimethyl modification of H3K27 (H3K27me3) serves as a docking site for PRC1 assembly and subsequent induction of higher chromatin organization (Figure 1) [5].

PRC1 consists of four core subunits, which are homologous to Drosophila Polycomb (Pc), Polyhomeotic (Ph), Posterior sex combs (Psc), and Sex combs extra (Ring/Sce). Each of these four core components has several orthologs in the mammalian genome [2, 20], which compete for incorporation into PRC1[21]. These orthologs are sorted into families of Cbx, Phc, Pcgf and Ring1 genes. The H3K27me3 repressive mark, established by PRC2, induces binding of PRC1 through the chromodomain-containing Cbx subunit [22,23]. Binding of PRC1 to chromatin allows mono-ubiquitination of histone 2A on lysine 119 (H2AK119ub1), which is stimulated by Pcgf and executed by Ring1 proteins [24]. H2A ubiquitination is believed to be the final step in stable gene silencing, as it blocks Pol II transcriptional elongation [3, 25-27].

Polycomb complex evolution

The emergence of multiple Polycomb-encoding genes during evolution coincided with the requirement for compaction of genetic information as genomes and multicellular organisms became more complex [28-29]. PcG genes expanded by multiple gene duplications and subsequent diversification of the ancestral gene [28-29]. In addition, an accumulation of single-nucleotide polymorphisms and the advent of new regulatory elements resulted in small changes in the DNA sequence and the emergence of alternative splice variants [28-29]. For example, in mammals four alternative splice-variants exist for the PcG gene Eed [8], and recently a new Cbx2 isoform has been discovered that lacks a polycomb repressor domain [30]. The

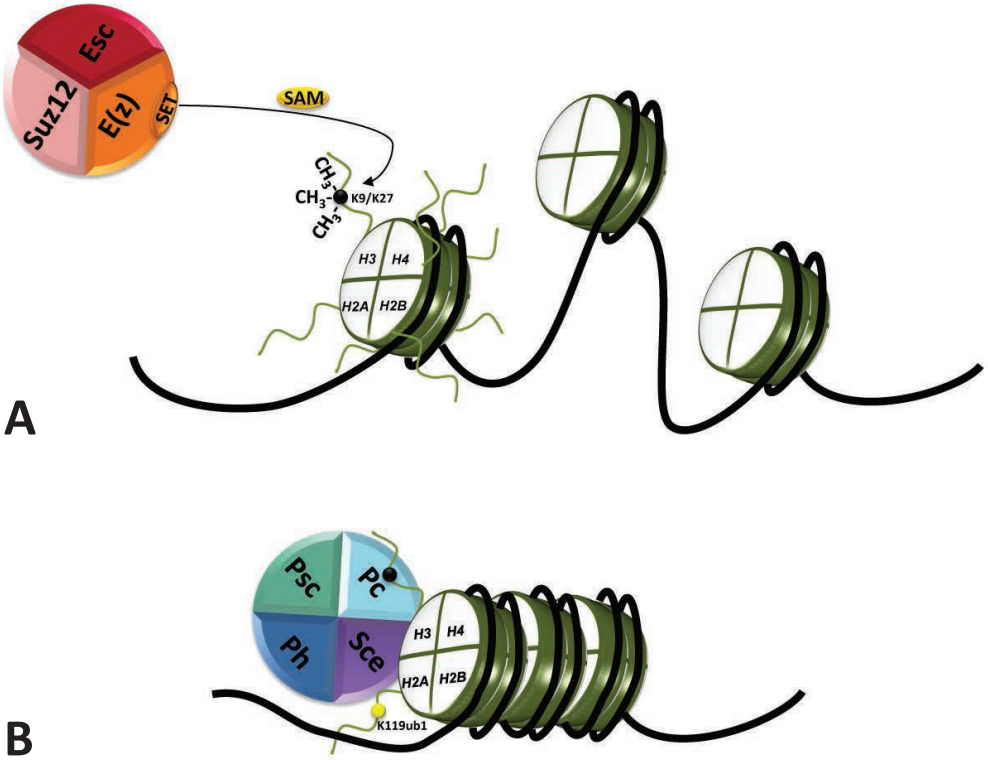

Figure 1. The canonical Polycomb-mediated gene silencing model. A PRC2 initiates gene repression by methylation of H3 on lysine 9 and 27 (H3K9/H3K27) catalyzed by the histone methyltransferase activity of the SET domain-containing E(z) subunit using the S-adenosyl methionine (SAM) co-factor as a donor for methyl groups. **B** The H3K9/27me3 histone marks are specifically recognized and bound by chromodomain-containing Pc subunits of PRC1 complexes. Binding of PRC1 to chromatin can drive further gene repression by mono-ubiquitination of H2A on lysine 119 (H2AK119ub1) by the Sce/Ring subunit.

expansion and divergence of different PcG genes ultimately resulted in large Polycomb gene families in mammals, such as the Pcgf and Cbx gene families. This resulted in multiple PRC sub-complexes, which can consist of various PcG orthologs.

PRC2 first emerged during early eukaryotic specification. It is possible that this occurred in the last common unicellular ancestor of eukaryotes, since several important protein domains of PRC2 members are conserved between plants and animals. RNAi-mediated silencing of the E(z) homolog in unicellular algae *Chlamydomonas reinhardtii* suggested that one of the primary roles of PRC2 in early eukaryotes was to maintain genome stability by suppressing transposable elements [31]. This suppression might have protected cells against adverse horizontal gene transfer. In multicellular organisms, their function might have been

adapted to regulate cell identity. The largest expansion of the PRC2 gene family occurred during plant evolution. Plants have up to 8 PcG members, which can form an array of PRC2 sub-complexes [32-33]. In contrast to plants, PRC2 members underwent little duplication in animals, with only four genes encoding for the core subunits (Eed, Ezh1, Ezh2, and Suz12) [31,34].

PRC1 homologs have not been identified in plants. However PRC1-like complexes (LHP1-AtRING1/LHP1-AtRING2) were shown to be involved in gene repression by binding to H3K27me3. This suggests that PRC1 function might have been conserved as a result of convergent evolution [32-33]. In contrast to PRC2 genes, expansion of PRC1 genes did occur in the animal kingdom, mainly at the invertebrate-vertebrate transition [28,35]. Although teleost radiation is accompanied by divergence of PRC1 members, further evolutionary events resulted in both expansion and loss of individual members. A combination of specific PcG genes might have increased the fitness of its carriers under certain environmental conditions and contributed to the evolution of tetrapods. (see Figure 2, Whitcomb *et al*. [28]; and Table 1, Le Faou *et al*. [35]) [29].

PcG diversification and functional complexity of PRC1 and PRC2

Functional complexity of PRC1

Due to this evolutionary diversification of PcG genes and proteins, a wide array of combinatorially distinct PRC complexes can occur in any cell. For example, five Cbx family members exist in mammals. Cbx2, Cbx4, Cbx7 and Cbx8, but not Cbx6, are known to function within the PRC1 complex [21]. Although the N-terminal chromodomain and C-terminal polycomb repressor box of Cbx proteins are highly conserved (typically >75% amino acid similarity), their affinity towards histone repressive marks (H3K27me3/H3K9me3) and PRC1 interacting partners varies substantially [21,23]. This distinct affinity might be attributed to high divergence in additional domains and motifs that have been acquired during evolution [28,29]. PRC1 can induce opposing activities, depending on which Cbx protein is present in the complex. For example, Cbx7 sustains pluripotency of embryonic stem cells (ESC), whereas other Cbx orthologs induce ESC differentiation and are directly repressed by Cbx7 [36-37].

A recent study identified six distinct PRC1 sub-complexes, each containing different Pcgf family members, and showed that these Pcgf sub-complexes bind differentially throughout the genome with little overlap in target genes [38]. This clearly suggests that different PRC1 sub-complexes have different molecular functions. Indeed, two Pcgf family members, Bmi1 and Mel-18, have been shown to have non-redundant functions at the molecular level, even though they share 70% of their protein sequence. Although integration of Bmi1 in PRC1 complexes enhances Ring1b E3 ligase activity, Mel-18 integration does not [24], indicating that these two orthologs have different molecular functions in chromatin compaction. An

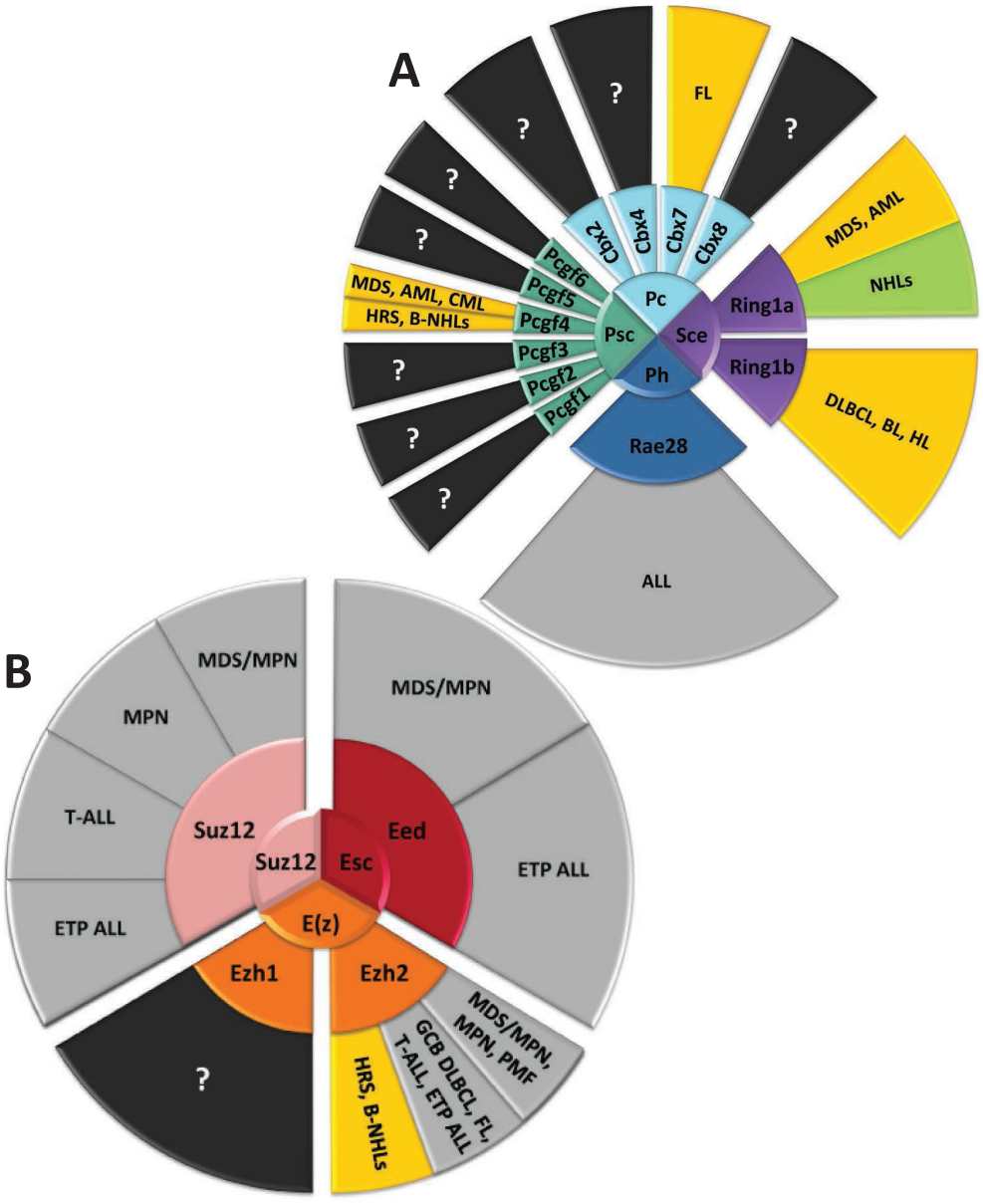

Figure 2. Schematic overview of PRC1 (A) and PRC2 (B) complex compositions and aberrations of various PRC members found in human hematological neoplasms. Diseases in the gray fields are caused by mutations, whereas diseases in the yellow fields are caused by overexpression of the corresponding Polycomb gene. Green fields represent single nucleotide polymorphisms (SNPs) in the corresponding Polycomb gene that correlated with a higher incidence of that particular disease. Black fields with the question mark indicate that no alteration in that particular PcG gene sequence or expression has yet been implicated in human hematological neoplasms.

Table 1

Complex		*Drosophila* gene	Mammalian Gene	Alias	Expression Alteration	Mouse Phenotype	Ref.
PRC1		Psc	Pcgf1	Nspc1, Rnf3a-2, Rnf68	NA	NA	
			Pcgf2	Mel-18, Rnf110, Zfp144	knockout/knockdown	3-6 weeks perinatal lethality; hypoplasia of spleen and thymus (defects in B-cell production)	41, 63, 67, 68
			Pcgf3	Rnf3, Rnf3a	NA	NA	
			Pcgf4	Bmi1, Bmi-1	knockout/knockdown	4-6 weeks perinatal lethality; impaired HSC self-renewal; hypoplasia of spleen and thymus	63-65, 71, 72
					overexpression	enhanced HSC activity	63
			Pcgf5	Rnf159	NA	NA	
			Pcgf6	Mblr, Rnf134	NA	NA	
		Pc	Cbx2	M33, Hpc1, Mod2, Cdca6, Srxy5	knockout/knockdown	5-6 weeks perinatal lethality; maintenance of HSC activity; hypoplasia of spleen and thymus	63, 77-79
					heterozygosity for a null allele/hypomorphic gene	no effect on HSC function	77
					overexpression	reduced HSC activity	63
			Cbx4	MPc2, HPc2	NA	NA	
			Cbx7	-	NA	NA	
					NA	Na	
			Cbx8	Pc3, Hpc3	knockout/knockdown	no effect on HSC activity	120
		Sce/Ring	Ring1	Ring1a, Rnf1	NA	NA	
			Rnf2	Ring1b, Ding, Ring2, Bap1	knockout/knockdown	early embryonic lethal	72, 86
		Ph	Phc1	Rae28, Edr, Edr1, Mph1, Hph1	knockout/knockdown	3-6 weeks perinatal lethality; reduced HSC activity; hypoplasia of spleen and thymus	84, 85
			Phc2	Ph2, Edr2, Mph2, Hph2, p36	NA	NA	
			Phc3	Edr3, Hph3	NA	NA	
	Pho-RC	Pho	Yy1	Nf-e1, Ucrbp, Delta, Ino80s	knockout	peri-implantation lethality	87, 141
					heterozygosity for a null allele (Yy1+/-) in Mpl-/- genetic background	reduced HSC activity	87
					overexpression	enhanced HSC activity; impaired lymphoid differentiation	88
		Sfmbt	Sfmbt1	Ru1	NA	NA	
PRC2		E(z)	Ezh1	Enh-2, Kmt6b	NA	NA	
			Ezh2	Enx1, Enx1h, Kmt6, Kmt6a, Wvs2	knockout/knockdown	early embryonic lethal; enhanced HSC activity; hypoplastic thymi; impaired lymphoid differentiation	49, 92, 93
					overexpression/knockin	maintenance/enhanced HSC activity; myeloproliferative disorder; splenomegaly	90, 91
					heterozygosity for a null allele ($Ezh2^{Del/+}$) in $Mpl^{-/-}$ genetic background	enhanced HSC activity	87
		Esc	Eed	l(7)5Rn, l7Rn5, Heed, Wait1	knockout/knockdown	early embryonic lethal	142
					heterozygosity for a null allele/hypomorphic gene	myelo- and lymphoproliferative defects	94
					heterozygosity for a null allele ($Eed^{3354/+}$) in $Mpl^{-/-}$ genetic background	enhanced HSC activity	87
		Suz12	Suz12	Chet9, Jjaz1	knockout/knockdown	early embryonic lethal	143
					heterozygosity for a null allele/hypomorphic gene	enhanced HSC activity	95
					heterozygous for a loss-of-function point mutation ($Suz12^{Pt18/+}$) in $Mpl^{-/-}$ genetic background	enhanced HSC activity	87

The following acronyms were used in this table: NA; Not Available

opposite effect of Bmi1 and Mel-18 on the cell cycle has also been demonstrated. Using a retroviral insertional mutagenesis approach in predisposed Eμ-Myc transgenic mice, Bmi1 was characterized as a proto-oncogene that collaborates with c-Myc [39]. It induces S-phase entry by inhibiting the function of retinoblastoma protein (Rb) through repression of the Ink4a/Arf locus [40]. In contrast, its ortholog Mel-18 arrests the cell cycle before entry to the S-phase by repressing c-Myc, resulting in downregulation of several cyclin-dependent kinases [41,42]. Moreover, Mel-18 could function as a cell-cycle inhibitor by downregulating Bmi1 expression [42,43]. Accumulating evidence thus indicates that the activity of PRC1 is highly dependent on its exact molecular composition [4,37,38,44-46].

Functional complexity of PRC2

Ample evidence also indicates that PRC2 has different functions depending on whether Ezh1 or Ezh2 is present in the complex. The catalytic SET domain of both Ezh proteins is highly conserved [28], and both possess catalytic activity to mono-, di- and tri- methylate H3K27. Ezh1 and Ezh2 compete for binding to Suz12 and Eed [11,47] and are part of distinct PRC2 complexes. These Ezh2- and Ezh1-containing PRC2 complexes appear to have complementary transcriptional repressive functions in ESCs [11,47]. Depletion of Ezh2 or Ezh1 shows differential effects on the level of methylated H3K27 in ESCs. H3K27 methylation was severely impaired upon Ezh2 depletion, whereas knockdown of Ezh1 did not result in global change of H3K27 methylation levels [11]. However, depletion of Ezh1 in $Ezh2^{-/-}$ ESCs abrogated residual methylation on H3K27, resulting in derepression of H3K27me3 target genes [47]. Thus, both Ezh1- and Ezh2-containing PRC2 complexes are necessary for proper H3K27me3-mediated silencing of target genes in ESCs. Functional complementation of Ezh1 and Ezh2 has also been observed in skin stem cells and in adult, but not in fetal liver, hematopoietic stem cells. In skin, ablation of both Ezh1 and Ezh2 resulted in reduction of H3K27me3 levels, arrested skin development and degeneration of hair follicles, while ablation of a single Ezh ortholog had no effect [48]. In fetal liver HSCs, conditional deletion of Ezh2 strongly compromised their function. However, in adult HSCs, Ezh1 is highly expressed and can compensate for the loss of Ezh2 and restore H3K27me3 levels [49].

Very recently, a novel role of Ezh1 became apparent in the regulation of transcriptional activation. In primitive skeletal muscle cells, Ezh1 can promote RNA polymerase elongation at gene bodies. In these cells, Ezh1-containing PRC2 complexes preferably associate with the active histone mark H3K4me3, while Ezh2-containing PRC2 complexes show canonical H3K27me3 association. Consequently, the overlap between target genes for Ezh2 and Ezh1 is very limited, and Ezh1- and Ezh2-containing PRC2 complexes show highly opposing roles in myoblast regulation [45].

A non-canonical model of PRC1 function

The canonical Polycomb-mediated model of gene silencing implies the hierarchical recruitment of PRC2 and PRC1 complexes at target sites. PRC2 initiates gene repression by methylation of H3K9 or H3K27, after which PRC1 is recruited by binding of the Cbx protein to this histone modification. PRC1 then drives further gene repression by ubiquitination of H2AK119. However, this model has recently been challenged on two grounds. First, PRC1 and PRC2 gene targets are not completely overlapping [50,51]. Non-overlapping genes targeted by PRC2 therefore lack ubiquitination of H2AK119. Second, disruption of PRC2 (partial or otherwise) does not prevent binding of PRC1 and ubiquitination of H2A on target genes, although the methylated histone mark is lacking [44,46,52].

A non-canonical PRC1 complex apparently exists, which could explain, at least partially, the functional independence of PRC1 from PRC2 and the H3K27me3 mark. Whereas the canonical PRC1 complex contains a Cbx protein, the non-canonical PRC1 complex contains either Rybp or Yaf2 [38,46]. Cbx proteins (Cbx2, Cbx4, Cbx7 or Cbx8) are mutually exclusive for PRC1, [21] and they specifically assemble in PRC1 complexes containing either Bmi1 or Mel-18, but not in PRC1 complexes containing other Pcgf orthologs [38]. These canonical PRC1 complexes bind H3K9me3 and/or H3K27me3 repressive marks set by PRC2 complexes. Strikingly, both canonical and non-canonical complexes have similar H2A ubiquitinating activity [46].

Polycomb PhoRC complex and recruitment of Polycomb complexes to chromatin

The diversity of PRC sub-complexes most likely results in targeting different collections of genes. In *Drosophila*, the PRC complex is recruited to DNA elements called Polycomb Response Elements (PREs) through interaction with the DNA-binding protein Pho [34,53]. Pho forms a stable two-subunit complex with the protein Sfmbt1 (Scm-like with four MbT domain-containing protein 1), which is referred to as the Pho-RC [3,14,54].

In *Drosophila* PhoRC plays an essential role in PRC recruitment, but no central recruitment mechanism or specific DNA elements have been identified in mammals. A first attempt by Ku *et al*. [51] using ChIP sequencing of PRC1 and PRC2 binding sites in ES cells did not result in the detection of specific sequence motifs, although PRC2-targeted sequences were found to be highly enriched in CpG islands. More recently, two studies reported the identification of mammalian PRE-like elements based on PcG recruitment. These PRE-like elements were found only at very specific sites (between the human Hoxd11 and Hoxd12 genes and upstream of the mouse MafB gene), and not throughout the genome [55]. These regions apparently do not qualify as consensus mammalian PRE elements. Both studies also suggested an important role for the mammalian transcription factor Yy1, the mammalian homolog of *Drosophila* Pho [22], in PRC recruitment. Multiple reports have now confirmed this

hypothesis [53,56]. However, since Yy1 and Polycomb target genes do not show a high degree of overlap [56], and Yy1 also has many PcG-independent functions in the cell [57], Yy1-mediated PRC recruitment is most probably not general, and additional mechanisms in mammalian cells are likely to exist. Solid evidence of the interaction of Polycomb complexes with other transcription factors has been lacking, but recent studies have shown that the transcription factors Gata1 [58], Hic1 [59], Rest [60], and the Runx1/CBFβ transcription factor complex [61] can also recruit Polycomb complexes to specific target genes. Besides transcription factors, long non-coding RNAs have also been recognized as important Polycomb recruiters [3,34]

More work is needed to address several remaining issues of PRC recruitment. For example, distinct Polycomb sub-complexes might utilize different recruiting mechanisms, thus binding different collections of genes; the non-canonical PRC1 complex, but not the canonical Cbx-containing PRC1 complex, may utilize Yy1-mediated recruitment since it contains the Rybp protein that has been shown to physically interact with Yy1 [62].

POLYCOMB PROTEINS AND HSC REGULATION

Although we found no studies on the effects of distinct PRC compositions in the hematopoietic system, hematopoietic phenotypes based on the ablation or overexpression of single PcG genes have been reported frequently (Table 1). Modulating PcG expression, thereby changing its protein abundance, is likely to alter the composition of PRCs, since PcG orthologs compete for incorporation. Therefore, these studies provide insight into the function of distinct PRC complexes in HSCs.

PRC1 members and HSCs

Bmi1 and Mel-18

Although highly similar in protein structure, the Pcgf family members Bmi1 and Mel-18 have very different effects on the maintenance of self-renewal of HSCs [63]. Despite normal embryonic hematopoiesis, Bmi1-null mutant mice displayed postnatal pancytopenia and died within 2 months after birth [63-65]. Park *et al*. [64] showed that the number of HSCs in Bmi1$^{-/-}$ fetal liver was not affected, whereas the number of HSCs in adult bone marrow was severely reduced. However, transplantation of Bmi1$^{-/-}$ fetal liver cells resulted in only transient hematopoietic reconstitution. This is compatible with the notion that Bmi1$^{-/-}$ HSCs cannot maintain long-term hematopoiesis, which causes hematopoietic failure soon after birth [63-64]. Indeed, it is now well established that Bmi1 is required for HSC self-renewal, both in mice and humans (Table 1) [66.]

Although Bmi1$^{-/-}$ fetal liver cells are functionally defective [64], Mel-18$^{-/-}$ fetal liver cells show almost normal repopulating capacity of the hematopoietic compartment [63], but a defect in B-cells might indicate its potential role in more differentiated cells (Table 1) [41,67]. Another study even reported a slight increase in HSC self-renewal capacity of adult Mel-18-deficient BM cells [68]. It seems likely that upon depletion of Mel-18 in HSCs, PRC1 complexes exclusively incorporate Bmi1, which then establishes the self-renewal phenotype. The specific function of Bmi1 in stem cells and of Mel-18 in more differentiated cells coincides with their endogenous expression patterns. Whereas Bmi1 is found to be specifically expressed in immature hematopoietic cells, its ortholog Mel-18 becomes upregulated along with differentiation [69].

Bmi1 is one of the best studied PcG members and many attempts have been made to clarify how Bmi1 sustains HSC self-renewal. In Bmi1$^{-/-}$ mice, the Ink4a-Arf locus was shown to be partially responsible for the hematopoietic defects; a double deletion of Bmi1 and Ink4a-Arf largely rescued the phenotype [40]. This locus encodes two proteins, p16^{Ink4a} and p19^{Arf}. p16 functions as a cyclin-dependent-kinase-inhibitor and hampers cell cycle progression by activating the Rb pathway, while p19 is important for p21/p53-mediated cell cycle arrest and apoptosis [70]. Ineffective self-renewal in Bmi1-deficient HSCs has been widely attributed to the derepression of the Ink4a-Arf locus and subsequent induction of premature senescence [63-64, 66].

More recently, it was shown that Bmi1 not only protects HSCs against premature cellular senescence, but also safeguards their multipotency. In HSCs, Bmi1 represses lineage specification through reinforcement of bivalent domains (overlapping repressive H3K27me3 and activating H3K4me3 histone marks) at the lymphoid regulator genes Ebf1 and Pax5. Bmi1-deficient HSCs showed loss of H3K27me3 and H2Aub1 repressive marks, resulting in a monovalent active state (H3K4me3) of these key lymphoid loci. Therefore, downregulation of Bmi1 might have promoted early expression of lymphoid genes and accelerated lymphoid specification [71].

Another mechanism by which Bmi1 can manifest its self-renewal function is its protective effect against oxidative stress and DNA damage. In the absence of Bmi1, reactive oxygen species levels were found to be elevated, resulting in increased apoptosis and reduced HSC numbers and activity [72-73]. Bmi1 also affects DNA repair, which is crucial to maintain genetically stable HSCs. Not only Bmi1, but also other PcG members, were found to be recruited to sites with double stranded breaks (DSB) and contributed to the initial steps of DSB repair [74]. Whereas overexpression of Bmi1 protects against the effect of radiation [75], Bmi1 knockdown increases radiosensitivity and resulted in a severe accumulation of DNA damage [74]. The role of Polycomb proteins in the DNA damage response was recently

reviewed by Gieni RS *et al*. [76]

Future research should clarify how all these Bmi1-mediated effects on senescence, apoptosis, lineage specification, reactive oxygen species levels and DNA damage are integrated and collectively regulate HSCs functioning.

Other PRC1 members in HSCs

Besides Bmi1, other PRC1 components have also been studied for their role in HSCs, although less intensively (Table 1). Most understanding of the role of various orthologs from the Cbx family originates from knockout mice. Studies of Cbx2 and Cbx8-deficient mice showed that neither gene is required for normal hematopoietic stem and progenitor function, although *in vitro* data suggested a role for Cbx2 in lymphopoiesis (Table 1) [63,77-79]. Also, in mice deficient for Cbx7, no effect on hematopoiesis was reported [80]. In contrast, overexpression of Cbx7 in the lymphoid compartment showed enhanced lymphomagenesis, which appeared to be dependent on c-Myc [81]. To our knowledge, Cbx4-deficient mice have not yet been generated. Although Cbx-deficient animals do not show overt hematopoietic abnormalities, caution is warranted with respect to the interpretation of these studies. It is likely that the absence of a single Cbx protein can be compensated by incorporation of another Cbx ortholog into PRC1 complexes during development. Functional redundancy between Cbx orthologs may therefore obscure the interpretation of PcG function in these studies.

Rae28-deficient mice exhibited perinatal lethality [82], and heterozygous Rae28$^{+/-}$ mice showed a severe delay in B-cell development [83]. In addition, fetal liver Rae28$^{-/-}$ HSCs were unable to functionally reconstitute the hematopoietic compartment of lethally irradiated mice [84-85]. Together this indicates that Rae28$^{-/-}$ HSCs are unable to sustain hematopoiesis during embryonic development. However, the function of Rae28 in adult HSCs has not been investigated. Ring1b exhibited a dual function in hematopoietic cells depending on their stage of differentiation. It restricted progenitor cell proliferation and stimulated maturation of their progeny via regulation of p16Ink4a and cyclin D2 expression [86].

Since Yy1 is the only—non-canonical—PcG member with DNA binding capacity, it can target PRC1 and PRC2 complexes directly to chromatin. Majewski *et al*. [87] included this protein in a sensitized *in vivo* screen in which they systematically tested whether individual PcG genes are required for HSCs and progenitor activity. They showed that defects in canonical PcG genes (Bmi1, Mel-18, Ring1a, Ring1b, Phc1/Rae28, Phc2, or Cbx2), and a defect in the non-canonical Yy1 were both associated with HSC/progenitor cell defects. Therefore, it appears that all core PRC1 components and Yy1 enhance HSC activity. The positive effect of Yy1 on HSCs was confirmed in a recent study, which showed that overexpression of Yy1 resulted in accumulation of HSCs [88].

PRC2 members in HSCs

Orthologous components of PRC2 have different expression patterns and functions in HSCs. While Ezh2 was found to be ubiquitously expressed in human and mouse bone marrow and fetal liver cells [49,69], its ortholog Ezh1 showed specific expression in adult mouse HSCs (Lin-Sca+ cKit+) and was decreased upon differentiation [49].

Although only few studies have explored the role of Ezh1, Ezh2 has been widely studied in HSCs using both gain- and loss-of-function approaches. Using a "genetical genomics" approach [89] followed by functional studies, Ezh2 was identified as an important HSC regulator [90]. Repeated serial transplantations greatly impair the potential of HSCs to reconstitute the hematopoietic system of recipient mice, but overexpression of Ezh2 completely prevents the exhaustion of HSCs during serial transplantations (Table 1). An inducible Ezh2 knock-in mouse was recently generated, which confirmed that elevated Ezh2 expression indeed increases the self-renewal capacity of HSCs [91].

However, loss-of-function studies did not support a positive role for Ezh2 in HSCs, but instead suggested an effect in early lymphoid development. Ezh2-deficient mice had no obvious HSC phenotype [92], although B and T cell development and VDJ rearrangement was affected [93]. $Ezh2^{-/-}$ LSK fetal liver cells transplanted into irradiated recipient mice showed high engraftment ability, and a severe impairment of lymphoid lineage differentiation, but the myeloid lineage was not affected [49]. In experiments that tested the effect of Ezh2 in a sensitized $Mpl^{-/-}$ background, not only Ezh2, but also Eed and Suz12 were found to restrict HSC activity. Although other studies also documented inhibitory effects of Eed and Suz12 on HSCs and progenitors [94,95], this is difficult to reconcile with the studies of Kamminga *et al*. and Herrera-Merchan *et al*. [90,91] We hypothesized that Ezh2 gene dosage and/or the balance between PRC2 complexes, including either Ezh2 or its homolog Ezh1, are important for proper HSC functioning. In addition, upon Ezh2 depletion, Ezh1 might complement Ezh2 in PRC2 integration and contribute to the functional activity of adult HSCs. Indeed, a recent study by Iwama *et al*. showed that Ezh1 can compensate Ezh2 loss in adult HSCs, but not in fetal liver HSCs [49].

Abundant evidence now shows that PcG genes must be correctly expressed in HSCs. We hypothesized that this correct expression ensures the multipotency of HSCs. A seemingly moderate imbalance, for example caused by gain or loss of function of individual PcG components, results in pronounced HSC phenotypes.

The diversity of PRC sub-complexes may regulate the dynamic equilibrium between HSC self-renewal and differentiation, but it is unclear how PRC complexes composed of different subunits actually affect HSCs. Ideally, proteomic analysis of PRC complex compositions should be conducted after PcG gene expression modification.

POLYCOMB PROTEINS IN HEMATOPOIETIC NEOPLASMS

Paradigmatically, leukemia is considered to be the consequence of a multistep process ultimately leading to unrestrained cell proliferation. Aberrant activity of genes involved in cell-cycle regulation is one of the prerequisites for malignant transformation of cells; many malignant cells have lost their cell cycle checkpoint control mechanisms. Both proto-oncogenes and tumor-suppressor genes (for example Ink4a-Arf) are often deregulated in cancer cells, and both classes of genes have been found to be under the control of PcG proteins [63, 96-97]. Interestingly, the aberrant expression of PcG genes has frequently been detected in various types of cancer, including hematological neoplasms (Figure 2) [98-99]. In addition, mutations in PRC2-encoding genes have recently been suggested as a causative factor in several types of leukemia. So far, PRC1 mutations have not been described in patients with leukemia. Strikingly, some PcG components, such as Bmi1 and Ezh2, have been reported to have both oncogenic and tumor-suppressor activity. Below we describe the functional involvement of PcG proteins in various types of hematological neoplasms.

PRC1 members in hematopoietic neoplasms

Bmi1

Bmi1 has been linked to leukemogenesis ever since it was identified as a cooperating partner of c-Myc in the induction of B-cell lymphomas [100].

Overexpression of Bmi1 is commonly found in patients with myelodysplastic syndromes (MDS) [101-102], acute myeloid leukemia (AML) [103], chronic myeloid leukemia (CML) [104-105], and various types of lymphoma [106-107]. In addition, the expression of Bmi1 has been shown to correlate with disease progression (Table 2) [106-107]. No evidence has been reported indicating that overexpression of Bmi1 is sufficient to induce leukemia. However, studies in various leukemic mouse models suggested that Bmi1 might be an important collaborating factor in leukemic transformation. For example, Sall4, an oncogene implicated in AML [108], was found to bind directly to the Bmi1 promoter and induce its expression [109]. Moreover, mice that constitutively overexpress Sall4 developed leukemia and displayed upregulated Bmi1 expression, particularly after disease progression to AML [109]. In a HoxA9-Meis1 leukemia mouse model, Bmi1 was shown to be essential for maintenance of leukemic stem cells (LSCs). Hoxa9 and Meis1 are oncogenes that were shown to induce transformation of murine BM cells [110]. Bmi1$^{-/-}$ fetal liver cells transduced with Hoxa9-Meis1 were able to induce AML when transplanted into irradiated recipient mice, but failed to sustain leukemia in secondary recipients [111]. In a MLL-AF9 mouse model, Bmi1 was shown to play an essential role in malignant transformation of myeloid progenitors into LSCs [112]. The fusion gene MLL-AF9 was found to cause immortalization of granulocyte macrophage progenitors *in vitro*

Table 2

Complex	Gene	Aberration	Hematopoietic phenotype	Species	Ref.
PRC1	Bmi1	overexpression	MDS, AML, CML	human	101-105
		overexpression	HRS, B-NHLs	human	106, 107, 117, 144
		overexpression	B-ALL	BCR-ABL mouse model	116
		knockout	MPN (MF)	mouse (Ink4a-Arf$^{-/-}$)	97
	Cbx7	overexpression	FL	human	81
		overexpression	T- and B-lymphomas	mouse	81
	Cbx8	knockout/knockdown	loss of AML phenotype	MLL-AF9 mouse model	120
	Ring1a	overexpression	MDS, AML	human	102
		SNPs (rs2855429, rs213213)	NHLs	human	122
	Ring1b	overexpression	DLBCL, BL, HL	human	123
	Rae28	loss of heterozygosity	ALL	human	83, 124-126
		loss-of-function	B-ALL	mouse	83
PRC2	Ezh2	overexpression	HRS, B-NHLs	human	106, 107, 117, 144
		loss-of-function/deletion	MDS/MPN (CML, CMML), MPN (MF), PMF	human	133-135
		gain-of-function (Y641 mutation)	GCB DLBCL, FL	human	127, 128
		loss-of-function/deletion	T-ALL	human	131
		loss-of-function/deletion	ETP ALL	human	132
		knockout	lower AML incidence	MLL-AF9 mouse model	139
	Eed	loss-of-function (point mut (G255D))	MDS/MPN (CML)	human	136
		loss-of-function/deletion	ETP ALL	human	132
		knockout	loss of AML phenotype	MLL-AF9 mouse model	139
		heterozygosity for a null allele/hypomorphic allele	thymic lymphoma	mouse (Eed$^{3354/+}$,Eed$^{1989/+}$,Eed$^{1989/1989}$)	145, 146
	Suz12	loss-of-function (aUPD 17q; point mut (E610G) del(17q11.2))	MDS/MPN (CMML)	human	136
		loss-of-function/deletion	T-ALL	human	131
		loss-of-function/deletion	ETP ALL	human	132

The following acronyms were used in this table:

AML; Acute Myeloid Leukemia
APL; Acute Promyelocytic Leukemia
aUPD; acquired Uniparental Disomy
B-ALL; B-cell Acute Lymphoid Leukemia
B-NHLs; B-cell non-Hodgkin lymphomas
BL; Burkitt's lymphoma
CML; Chronic Myeloid Leukemia
CMML; Chronic Myelomonocytic Leukemia
ETP-ALL; Early T-cell Precursor Acute Lymphoblastic Leukemia
FL; Follicular Lymphoma
GCB DLBCL; Germinal Center B subtype of Diffuse Large B-Cell Lymphoma
HL; Hodgkin's lymphomas
HRS; Reed-Sternberg cells of Hodgkin's disease
MDS; Myelodysplastic Syndromes
MF; Myelofibrosis
MPN; Myeloproliferative Neoplasms
NHLs; Non-Hodgkin Lymphomas
PMF; Primary Myelofibrosis
T-ALL; T-Cell Acute Lymphoid Leukemia

and development of AML upon transplantation into irradiated recipient mice [113]. However, granulocyte macrophage progenitors derived from Bmi1-deficient mice and transduced with MLL-AF9 displayed reduced proliferative and clonogenic capacity *in vitro* and absence of leukemia development *in vivo*.

Several studies have shown that Bmi1 can play a role in the progression of CML towards acute blast crisis. The BCR-ABL oncoprotein is necessary and sufficient to initiate CML [114], but overexpression of Bmi1 was required to provoke the progression to a more advanced stage of the disease [104]. $CD34^+$ cells from CML patients transduced with Bmi1 showed enhanced proliferative capacity and self-renewal properties in vitro, and resulted in a transplantable leukemia *in vivo* [115]. Synergistic effects of Bmi1 and BCR-ABL have also been shown to trigger acute B-cell lymphoid leukemia (B-ALL) development in mouse models [116].

The above results indicate that Bmi1 cooperates with oncogenic fusion proteins in leukemic transformation. In human leukemias, Bmi1 protects LSCs from senescence and apoptosis by inhibiting p16 and p19 expression [96,111], but enhanced expression of Bmi1 may also result in protection of LSCs from oxidative stress66. Pharmacological downregulation of Bmi1 was therefore predicted to reduce the proliferative capacity of AML cells. This hypothesis was supported in an experimental study where downregulation of Bmi1 in AML $CD34^+$ cells reduced their proliferative capacity and stem/progenitor cell frequency by inducing ROS accumulation and apoptosis [66].

In contrast to the role of Bmi1 in promoting self-renewal of normal BM cells and its oncogenic role in several types of leukemia, a recent study by Oguro *et al*. [97] demonstrated a potential tumor-suppressor function of Bmi1 (Table 2). Irradiated recipient mice, repopulated by $Bmi1^{-/-}$ Ink4a-$Arf^{-/-}$ BM cells, developed lethal myelofibrosis (MF). These mice showed an expansion of LSK and myeloid progenitor cells, along with abnormal megakaryocytopoiesis in the bone marrow.

Other PRC1 members

Although a role for Bmi1 in leukemogenesis appears to be well established, less is known about other Pcgf family members. Several studies, based on immunohistochemical analyses of leukemic tissues, proposed that aberrant expression of PcG genes results in abnormal formation of PRC1 complexes and suggested that this might have contributed to the development of hematological neoplasms [117-118]. In a study where the expression of multiple Polycomb family members was assessed in 126 AML patients by real-time qPCR, Bmi1 was found to be overexpressed, whereas Mel-18 was one of the few tested PcG genes that showed no overexpression [119].

Little is known about the contribution of Cbx family members to the development of hematopoietic malignancies. Cbx8 was shown to be essential in MLL-AF9 induced leukemogenesis, even though this function was found to be PRC1-independent (Table 2) [120]. Cbx8 was also shown to be required for leukemic transformation of other MLL fusion proteins121. One study showed that Cbx7 is often overexpressed in human follicular lymphomas (FLs) [81] and that Cbx7 overexpression in the mouse lymphoid compartment results in T-cell and B-cell lymphomas upon transplantation (Table 2) [81]. Cbx2 and Cbx4 have not been associated with leukemia development.

The PRC1 member Ring1a is commonly overexpressed in MDS and AML and correlates with poor prognosis [102]. In addition, single nucleotide polymorphisms (SNPs) in Ring1a were found to be associated with non-Hodgkin lymphoma [122]. Enhanced expression of its ortholog Ring1b has been detected in lymphomas such as diffuse large B-cell lymphoma (DLBCL), Burkitt's lymphoma and Hodgkin's lymphomas (Table 2) [123].

The Rae28 locus was found to be disrupted in patients with hematological malignancies [83,124–126]. Furthermore, it was demonstrated that inactivation of Rae28 is implicated in severe B-cell maturation arrest (Table 2) [83].

PRC2 members in hematopoietic neoplasms

Similar to PRC1 components, aberrant expression of PRC2 genes, most notably Ezh2, has been associated with the development of hematopoietic neoplasms (Table 2). In 2000, it was first reported that increased Ezh2 expression might promote lymphoma development. [106,107,117] More recently, recurrent heterozygous Tyr641 mutations that occur in the catalytic SET domain of Ezh2 were discovered in two types of lymphomas that arise from germinal center B-cells. These mutations were found in approximately 20% of the patients with a germinal center B (GCB) subtype of DLBCL and in 7% of follicular lymphoma FLpatients [127,128]. While the recurrent heterozygous Tyr641 mutation of the Ezh2 allele resulted in a reduced ability to trimethylate H3K27 [127], co-occurrence with the wild type Ezh2 allele induced increased H3K27me3 levels. Heterozygous Tyr641 mutations therefore effectively represent a gain-of-function mutation [129]. This supports the hypotheses that Ezh2, which is often overexpressed in lymphomas, can contribute to the disease [107]. Very recently, pharmacological inhibition of the methyltransferase activity of Ezh2 by GSK126 was shown to effectively inhibit the proliferation of Ezh2 Tyr641 cell lines and DLBCL xenografts in mice [130]. This drug might therefore be a promising treatment for patients with activating mutations in Ezh2. However, caution is warranted when administering such inhibitory drugs to patients, since inactivating mutations and deletions of Ezh2 and other PRC2 components have also been discovered recently. In a study of 68 adult T-ALL cases, loss-of-function mutations and deletions of Ezh2 (18%) or Suz12 (7%) were found [131]. In a separate study,

the incidence of inactivating PRC2 mutations was found to be even higher in a pediatric subtype of T-ALL; 40% of ETP ALL patients showed a deletion or sequence mutation in Eed, Ezh2 or Suz12 [132]. Various inactivating homozygous and heterozygous Ezh2 mutations were also discovered in patients with myelodysplastic and myeloproliferative neoplasms [133,134]. Ezh2 mutations, which are associated with poor prognosis, were shown to coexist with the well-known Jak2V617F mutation in primary myelofibrosis [135]. Mutations in other PRC2 components (Eed and Suz12) have also been detected in MDS/MPN patients, albeit with lower frequencies (Table 2) [136].

The above findings indicate that loss or gain of Ezh2 activity (either by altered expression or by mutations) can both contribute to leukemogenesis in patients. These "two faces of Ezh2 in cancer" have been well described in a recent review by Hock *et al*. [137] The function of Ezh2 as both a tumor-suppressor and an oncogene has also been described in various mouse models [138,139]. We suggest that a single PcG can display either tumor-suppressor or oncogenic functions depending on the cell context and its interaction partners. Prescreening to determine whether mutations cause either a loss or gain of function is therefore crucial before starting PRC2-targeted therapy in patients.

CONCLUDING REMARKS

It has now become evident that PcG proteins play an important role in both normal hematopoiesis and in various hematological malignancies. Depending on the stage of hematopoietic cell differentiation when neoplastic transformation first occurs and which PcG gene is involved, different types of leukemia may arise. In addition, depending on the cell context and interacting partners, individual PcG genes may display either tumor-suppressor or oncogenic functions. Compositional rearrangements of PRCs may provide true oncogenic events, instead of aberrant PcG functions caused by mutations or misexpression. However, the fact that leukemic cells are genetically unstable, and are susceptible to additional genetic abnormalities, make it difficult to determine whether PcG aberrations are a cause or a consequence of malignant transformation.

It should be noted that besides PcG proteins, other epigenetic mechanisms, such as DNA methylation and ncRNA interference, are also employed in regulation of gene expression [140]. Understanding the interplay between these distinct epigenetic mechanisms in the maintenance of a cell-type specific epigenome will be important for leukemia research.

The assessment of the epigenetic signatures of normal and leukemic stem cells is of great importance for future cancer research since compositional changes of PRC complexes may

present useful prognostic markers. Pharmacological targeting of PRC compositions may therefore be an effective anti-leukemia strategy.

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Chapter 3

Polycomb group proteins in hematopoietic stem cell aging and malignancies

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ABSTRACT

Protection of the transcriptional “stemness” network is important to maintain a healthy HSCs compartment during the lifetime of the organism. Recent evidence shows that fundamental changes in the epigenetic status of HSCs might be one of the driving forces behind many age-related HSC changes and might pave the way for HSC malignant transformation and subsequent leukemia development, the incidence of which increases exponentially with age. Polycomb group (PcG) proteins are key epigenetic regulators of HSC cellular fate decisions and are often found to be misregulated in human hematopoietic malignancies. In this review, we speculate that PcG proteins balance HSC aging against the risk of developing cancer, since a disturbance in PcG genes and proteins affects several important cellular processes such as cell fate decisions, senescence, apoptosis, and DNA damage repair.

INTRODUCTION

The appearance of an aged individual is often typical; skin wrinkles, hair loss, and fragility often accompany loss of visual and auditory abilities and cognitive decline. These consequences of aging are associated with an increased risk for developing age-related diseases, such as cancer, neurodegenerative diseases and diabetes. Although the phenotypic characteristics of aging are evident, their molecular causes are not well understood. In general, aging of an organism must result, to a great extent, from tissue dysfunction and degeneration as a consequence of cellular aging. Mature cells of most tissue types are short-lived and need to be continuously replenished throughout life. This process is preserved by the enduring activity of tissue-specific adult stem cells. Hematopoietic stem cells (HSCs), which reside within the bone marrow, are the best studied category of adult stem cells [1-3]. These cells are able to undergo both symmetric and asymmetric divisions, leading to their self-renewal or differentiation. A precise coordination of these cell fate decisions is essential to sustain proper blood cell production throughout the entire lifespan of the organism.

In HSCs, multiple age-dependent changes have been identified. Successively serially transplanted HSCs from several strains of mice remained able to repopulate the hematopoietic system of recipient mice [4-6]. However, these cells were less functional than their young counterparts and eventually exhausted [6-10]. Likewise, studies in which HSCs isolated from old mice were directly compared to HSCs obtained from young mice clearly showed that HSC function declines with age. Old HSCs possessed impaired homing potential and self-renewal activity and, in addition, showed a propensity toward a myeloid-lineage-skewing of differentiation potential [11-12].

Dysfunction at the level of the stem cell compartment might underlie the decline of several age-related parameters in hematopoietic tissue function. For example, numerous impairments in the functioning of an aged immune system are found [13-14]. These might either be attributable to an impaired activity and specificity of mature T cells, B cells, macrophages and NK cells, or more directly related to bone marrow failure. The changes in the functional properties of HSCs might also have more severe pathological consequences. For example, it has been shown that with advanced age the incidence of hematological malignancies increases substantially. Myelodysplastic syndromes (MDS) are the most prevalent hematologic disorders in the elderly population. These syndromes are defined as clonal stem cell disorders characterized by ineffective differentiation. In patients this results into peripheral blood cytopenias in combination with a hypercellularity of the bone marrow. Ultimately, MDS often progresses into acute myelogenous leukemia (AML).

Although many theories have been put forward to explain the phenomenon of aging, studies in the mouse hematopoietic system have made it increasingly evident that there is a clear stem cell basis for age-associated changes. However, the molecular mechanisms driving functional changes in HSCs during aging remain elusive. First of all it is still a matter of debate whether aging is a programmed or stochastic process that occurs in all individual HSCs equally [11], or whether the clonal composition of the HSC compartment changes [12, 15-18]. Theoretically, the latter might ultimately result in a monoclonal dysfunctional HSC population. Second, oxidative and metabolic stress, telomere attrition and defects in DNA repair mechanisms all contribute to severe DNA damage which may result in an instable genome and the formation of toxic mutant proteins. Yet, neither of these processes in isolation can account for all age-related changes observed in HSCs. Thus, aging appears to be a multifactorial process.

There is emerging consensus that safeguarding the transcriptional "stemness" network is important to maintain a healthy HSC compartment. Gene profiling studies, for example, demonstrate that gene expression programs of aged hematopoietic stem cells are different from those in their young counterparts [12, 19-22]. Whether these changes are caused by genetic or epigenetic dysregulation of gene expression programs is still unclear. An accumulation of mutations in the DNA sequence and a dysfunctional DNA repair machinery may result in the observed changes in gene expression. However, changes in the chromatin status of the cell are also expected to have a strong causative effect in altering gene expression.

Factors that control chromatin organization, the manner in which the DNA is packed around histones, are important for safeguarding the genomic integrity as well as transcriptional activity of the cell. Several chromatin modifiers have shown to be essential in the regulation of HSC cell fate decisions [23-24]. Dysregulation of these epigenetic factors that control the balance between self-renewal and differentiation might account for many age-related impairments in HSC function. In this review we will discuss recent evidence that supports the concept that fundamental changes in the epigenetic status of HSCs might be one of the driving forces behind many age-related HSC changes.

AGE-ASSOCIATED EPIGENOMIC CHANGES IN HSCs

Epigenetic regulation of chromatin structure can be considered a gatekeeper of cellular memory, since it is crucial for establishing, maintaining and propagating transcriptional profiles of the cell [25-26]. Epigenetic modulation involves DNA methylation of cytosine residues in CpG islands [27] and modifications of histone tails, mostly by acetylation, methylation and ubiquitination [27-29]. During symmetrical divisions, daughter cells have to inherit the same

epigenetic status as the parental pluripotent cell to maintain the unique HSC cell fate. Thus, for a proper self-renewing cell division, besides the DNA sequence the chromatin structure also needs to be copied. In contrast, during asymmetrical divisions, chromatin modifications need not to be faithfully copied, leading to differential gene expression profiles and a distinct identity of both daughter cells.

Throughout life, cellular memory needs to be maintained, and proteins that control chromatin organization are therefore likely to be important for the maintenance of cell-type specific gene expression patterns during HSC aging as well [30-31]. This interpretation is supported by a study of Chambers and coworkers [19] in which gene expression profiles of HSC isolated from young (2 months), intermediate (6 and 12 months) and old mice (21 months) were compared. Genes involved in transcriptional silencing via chromatin regulation were specifically downregulated with age. These included histone modifying genes from the SWI/SNF-complex (Smarca4 and Smarcb1), the polycomb (Ring1) and Trithorax complexes (Mll3, Mllt10), histone deacetylases (Sirt2, Sirt3, Sirt7, Hdac1, Hdac5, Hdac6) and histone acetyl transferases (Myst2). Genes involved in DNA methylation were also found to be repressed during aging. These included DNA methyltransferase (Dnmt3b) and the methyl-CpG-binding protein encoding genes (Mbd1). In a related gene expression study by Rossi and colleagues, the PcG gene Ezh2, which possesses histone-methyl-transferase activity, was found to be downregulated in old HSCs, while Smarca2 and polycomb-interacting protein Jarid1a were found to be significantly upregulated [21].

In a recent study conducted with human cells, genes involved in chromatin remodeling were also overrepresented in being differentially expressed in old human hematopoietic stem/progenitor cells (HSPCs), compared to their young counterparts. Using micro-arrays, gene expression profiles from old HSPCs isolated from mobilized blood from individuals between 27 and 73 years were compared to gene expression profiles from young HSPCs isolated from cord blood [22]. The polycomb genes Ezh1 and Cbx7 and polycomb-associated gene Jarid2 were upregulated in old human HSPCs. Upregulation was also observed for other chromatin-modifying genes such as Rnf20, Jmjd1a, Mllt4 and Sirt2. Other chromatin regulators such as Hdac6, Ezh2, Hmgn2, Myst4, Smarca5, Cbx1 and Cbx5 were found to be significantly downregulated with age.

Together, these studies imply that genes involved in chromatin organization are frequently dysregulated upon HSC aging. In addition, both in old murine HSCs and human HSPCs, large gene clusters were identified that were coordinately up or down-regulated. This may reflect the impact of dysregulated epigenetic factors on genome-wide expression profiles [19, 22]. In agreement with this hypothesis, in thirteen different tissues it has been shown that genes that were normally coordinately expressed in young to intermediate-aged tissues (from 16

month mice), failed to do so in older tissue (24 months) [32]. Moreover, aberrantly expressed genes tended to be clustered in close proximity to each other. Together, these observations support the concept of a deterioration of chromatin integrity with age (Figure 2).

Due to the difficulty of collecting adequate cell numbers for analysis, genome-wide studies on chromatin modification in aged HSCs are so far lacking. However, studies in other cell types have shown that several epigenomic changes indeed accumulate over time [33-35]. Although overall genomic DNA methylation has been found to decrease with age, a number of specific promoters become hypermethylated. Intriguingly, both genome-wide DNA hypomethylation and aberrant promoter hypermethylation are also epigenetic hallmarks of cancer [36-37], which will be discussed further in this review. An age-associated decrease in histone acetylation and alterations in histone methylation patterns have also been reported [33].

In concert, chromatin dysregulation might present a plausible explanation for the numerous and broad genome-wide changes in gene expression levels observed in aged HSCs.

POLYCOMB GROUP PROTEINS AND HSC REGULATION

PcG proteins

Polycomb group (PcG) proteins form an explicit class of epigenetic modulators. They assemble within multimeric protein complexes and induce transcriptional repression of target genes through catalyzation of specific histone modifications. PcGs were originally identified in *Drosophila* as repressors of homeotic (Hox) genes [38]. Together with the counteracting *Trithorax* group (TrxG) proteins, PcGs showed to be crucial for maintenance of appropriate patterns of Hox gene expression for correct axial patterning of body segments. The function of PcG proteins seemed to be highly conserved during evolution since they also showed to be important for proper development in mammals. For example, several mutant mice models that were generated displayed skeletal malformations [39-42].

Biochemical analyses have revealed that PcG proteins assemble within at least two multimeric protein complexes, termed Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2) (Figure 1) [26, 43-44]. PRC2 contains three core subunits homologous to Drosophila; Enhancer of zeste (E(z)), Suppressor of zeste 12 (Suz12) and Extra sex combs (Esc). The currently identified mammalian orthologs that have shown to function within PRC2 are Ezh1/Ezh2, Suz12 and Eed [45-48]. In addition, three different isoforms exist for Eed (Eed1, Eed2 and Eed3/4 [49-50]. The SET-domain containing Ezh proteins catalyze trimethylation of histone 3 lysine 27(H3K27me3) and to a minor extent lysine 9 of histone 3 (H3K9me3). This modification is found to be the initiating step of gene repression.

PRC1 complexes consist of four core components; *Drosophila* polycomb (Pc), Sex combs extra (Ring/Sce), Polyhomeotic (Ph), and Posterior sex combs (Psc) proteins (Figure 1). Each of these proteins have several orthologs in mammals, classified respectively as the Cbx, Ring1, Phc, and Bmi1/Mel18 families. Cbx proteins recognize and catalyze PRC1 binding to the H3K9/27me3 mark which has been established by PRC2 [51-52]. Subsequently, Ring1 possesses histone 2A ubiquitination activity [52-54]. Data indicate that this histone modification is the final step in gene repression since it blocks the movement of RNA polymerase along the DNA [55-56]. However, several studies challenge this strict hierarchical recruitment model, since PRC1 and PRC2 can also function independently [57-60].

The number of genes encoding PcG family members has expanded tremendously in mammals, which resulted in a great combinatorial diversity in PRC1 and PRC2 complex compositions (Figure 1) and molecular functions. For example, the Cbx family consists of Cbx2, Cbx4, Cbx6, Cbx7 and Cbx8. The chromodomains of different Cbx proteins were found to have different affinities toward mono-, di-, and tri-methylated H3K9 and H3K27 [51]. Likewise, whereas Bmi1 can stimulate the E3 ubiquitin ligase activity of Ring1b, its homologue Mel18 does not possess this ability [53]. Distinct functional roles for Ezh1- and Ezh2-containing PRC2 complexes in gene repression were found as well [61]. Taken together, stoichiometric perturbation of one component of the PRC complex might be sufficient to modify its molecular function.

As discussed before, expression levels of several PcG genes showed to be affected during HSC aging [19, 21-22]. One can imagine that changes in gene expression levels of PcG genes during HSC aging results in modifications of the compositions and diversity of PRC1 and PRC2 complexes. The observed age-related changes in HSCs, such as the myeloid-skewage of differentiation and reduced self-renewal abilities, might be the result of a misbalance in HSC cell fate decisions by PcG misregulation and/or altered ratios in terms of PcG homologs (Figure 2).

PcG proteins in HSC regulation

Polycomb group proteins have shown to be key players in HSCs by determining cellular fate decisions (Figure 1). In HSCs, they repress the transcription of lineage-specific genes. In response to extrinsic or intrinsic signals, PcG proteins can be displaced from these promoters and be recruited to stem cell-specific self-renewal genes to promote differentiation and suppress unlimited proliferation. However, during differentiation into either the myeloid or lymphoid lineage, PcGs not only repress the transcription of self-renewal genes but also of differentiation genes of the alternative lineage.

The precise mechanism whereby PcGs are recruited to their target loci remains elusive, although evidence accumulated that several transcription factors and long non-coding RNAs are involved in this process [62]. Interestingly, one recent report showed that long non-coding

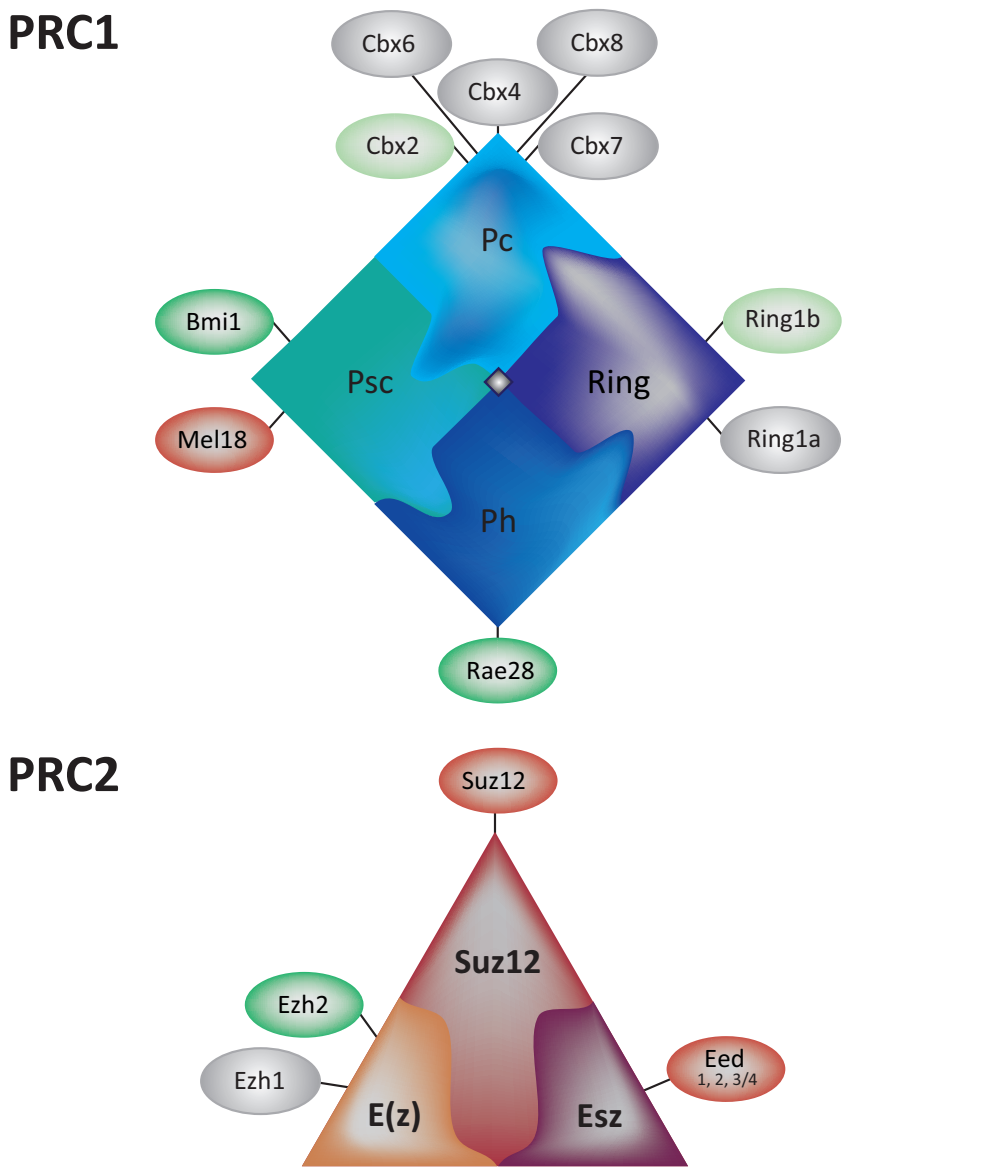

Figure 1. Schematic representation of the two Polycomb Repressive Complexes and their role in Hematopoietic Stem Cell regulation. The PRC1 complex consists of four core subunits homologous to Drosophila Polycomb (Pc), Sex combs extra (Ring), Polyhomeotic (Ph), and Posterior sex combs (Psc). The PRC2 complex comprises three core subunits, Enhancer of zeste (E(z)), Suppressor of zeste 12 (Suz12), and Extra sex combs (Esc). In mammals, several different homologs exist for every distinct PRC core subunit, resulting in PRC complex composition diversity. Expression pertubation studies showed that distinct PcG genes have different functions in Hematopoietic Stem Cell regulation. Several PcG genes stimulate HSC or progenitor self-renewal divisions (dark and light green balloons, respectively), whereas others negatively affect HSC self-renewal (red balloons). Several PcG genes have not yet been studied for their role in HSC regulation (grey balloons).

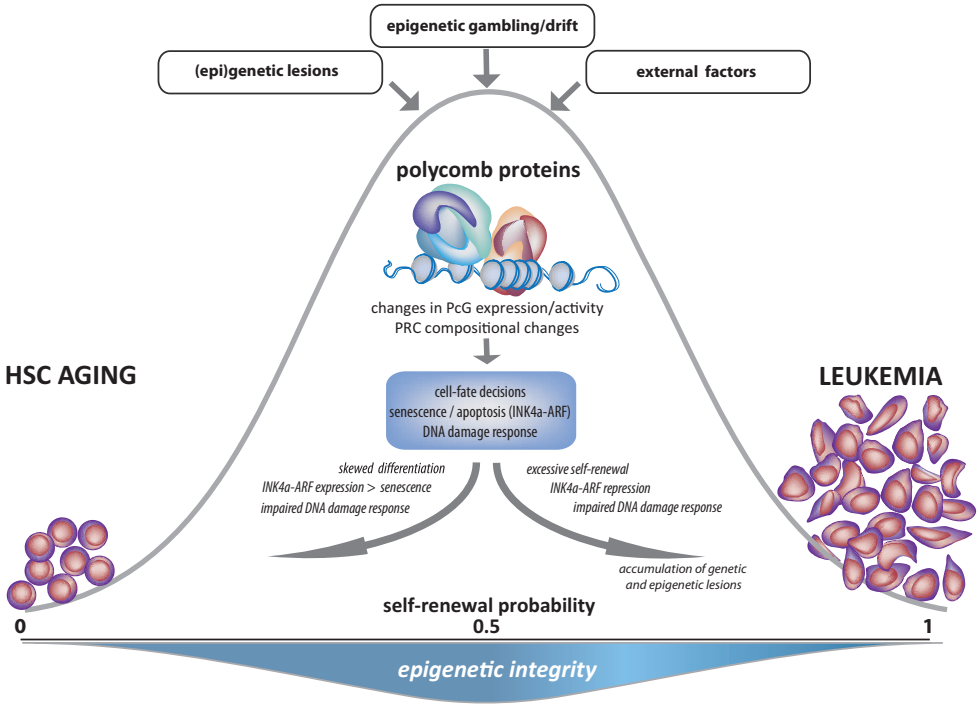

Figure 2. Model in which Polycomb Group proteins balance HSC aging and cancer development. External factors, genetic or epigenetic lesions or stoichiometric changes in expression or activity can alter the function of Polycomb Group proteins and the composition of the Polycomb Repressive Complexes. Polycomb group proteins are known to safeguard the epigenetic integrity of the cell. However, misregulation of Polycomb Group proteins can affect several important cellular processes such as cell-fate decisions, the p16/p19 senescence and apoptosis pathway, and the DNA damage repair machinery. Impaired self-renewal activity, a differentiation skewage and derepression of the INK4a-ARF locus which results in HSC senescence, are common hallmarks of aging. However, malignant transformation of HSCs is generally associated with the acquisition of excessive self-renewal properties by, for example, stable repression of the INK4-ARF locus by Polycomb Group proteins and improper functioning of the DNA damage response machinery. This might subsequently promote accumulation of genetic and epigenetic errors and further drive leukemic progression.

RNAs (lncRNA) are able to physically connect polycomb complexes with another histone modifying complex (LSD1/coREST/REST) to coordinately specify histone modifications on target genes [63]. LSD1 specifically catalyzes H3K4 demethylation. Whereas polycomb-mediated H3K27me3 is generally associated with repressive chromatin, methylated H3K4 is considered to be an active histone mark. Only recently, the existence of bivalent marks (the appearance of both “active” methylated H3K4 and “repressive” methylated H3K27 marks on the same locus) has been reported in murine CD150+ LSK HSCs and human $CD34^{+}$ CD133+ HSCs [64-65]. In embryonic stem cells, bivalent chromatin marks are thought to keep

developmental genes poised for activation. Oguro *et al*. [66] recently discovered that Bmi1 is involved in transcriptional repression of lymphoid loci (Pax5 and Ebf1) by bivalent domains in hematopoietic stem cells. The physical interaction of polycomb complexes with an H3K4 demethylase complex mediated by lncRNA might be important for the removal of bivalent domains in HSCs upon differentiation. However, this concept needs further experimental support.

Many PcG genes show stage-specific expression patterns along hematopoietic differentiation. For example, Bmi1 is preferentially expressed in HSCs and its transcript levels decrease upon lineage specification, whereas Eed shows ubiquitous expression in all hematopoietic stages [67-69]. By analysis of the subcellular localization of PcG proteins in different hematopoietic cell populations, LT-HSCs (CD34- LSK), multipotent progenitors ($CD34^+$ LSK) and B cell progenitors (B220+ CD43+), Kato *et al*. [70] showed that PRC1 complexes in primitive HSCs showed a unique composition different from progenitor cells.

HSC regulation by PRC1 members

Bmi1 is the first discovered and best studied polycomb member involved in HSC regulation (Figure 1). By a retroviral insertional mutagenesis approach in predisposed Eμ-myc transgenic mice, Bmi1 was originally discovered as a proto-oncogene that cooperates with MYC in the generation of B and T cell lymphomas [71]. The importance of Bmi1 in adult hematopoiesis became particularly apparent in Bmi1-null mutant mice [42]. Despite normal embryonic development, these mice showed hypoplastic bone marrow and reduced white blood cell counts due to impaired bone marrow hematopoiesis after birth. Park *et al*. showed that the number of HSCs in $Bmi1^{-/-}$ fetal liver was not affected but the number of HSCs in postnatal $Bmi1^{-/-}$ mice was severely reduced [72]. Bmi1-deficient mice died within two months of birth. Functional analysis of $Bmi1^{-/-}$ HSCs in a transplantation setting showed that these cells were incapable of self-renewal since they did not sustain long-term hematopoietic repopulating ability [67, 72]. In turn, forced expression of Bmi1 in CD34- LSK sorted HSCs promoted self-renewal [67]. This effect was restricted to HSCs, since overexpression of Bmi1 in multipotent progenitors (CD34- LSK) gave no such effects [67]. Recently, the important role of Bmi1 in human HSCs ($CD34^+$ cord blood cells) was confirmed [67, 73-74].

The PRC1 component Mel18, a homologue of Bmi1 with 70% identity at the amino-acid level, showed negative effects on the self-renewal activity of HSCs (Figure 1) [75-76]. Recently, some evidence has emerged showing that the balance between Bmi1 and Mel18 regulates HSC functioning [76]. However, this concept needs further support, for example by studying whether Bmi1 and Mel18 indeed compete for incorporation in the PRC1 complex in HSCs.

Other PcG PRC1 components are also studied for their role in adult HSC regulation, although less intensively (Figure 1). Inactivation of Ring1a and Ring1b in mice showed distinct phenotypes. Ring1a $^{-/-}$ mice are fertile but show a number of minor skeletal abnormalities along the anterior-posterior axis [41], whereas Ring1b $^{-/-}$ mice showed defective gastrulation and were embryonic lethal [77]. In the hematopoietic system, conditional deletion of Ring1b resulted in hypocellularity of the bone marrow. Ring1b was found to specifically restrict the proliferation of progenitors and it paradoxically promoted proliferation of their maturing progeny. Thus, Ring1b showed to have dual functions in the regulation of hematopoietic cell turnover depending on the maturational stage of cell differentiation by both targeting cell cycle activators (cyclin D2 and cdc6) and inhibitors (p16 ^{Ink4a}) [78]. PRC1 member Rae28 (a.k.a. Phc1, Edr1, Mph1) is the only member of the Phc family that has been studied for its role in hematopoietic stem cells. During embryonic development, the number of HSCs in fetal liver progressively declines in Rae28-deficient mice [79]. Rae28 showed to be essential for effective HSC self-renewal and Rae28$^{-/-}$ stem cells were unable to reconstitute the hematopoietic compartment upon serial transplantation experiments [79-80].

At present, the Cbx family has been the least studied PcG PRC1 member with respect to their role in HSCs (Figure 1). The Cbx family of proteins comprises Cbx2, Cbx4, Cbx6, Cbx7 and Cbx8, and all can bind to H3K27me3 marks through their N-terminal chromodomain. Cbx $^{-/-}$ mice showed normal numbers of HSCs in fetal liver and these HSCs were fully functional even after successive serial transplantations [67]. Although no gross abnormalities on fetal liver HSCs number and function was observed, 4-week-old Cbx2$^{-/-}$ mice showed several lymphocyte abnormalities [40]. By means of *in vitro* proliferation assay using thymidine incorporation, Cbx2 appeared to be required for T cell precursor proliferation [40]. This is further supported by the observation that Cbx2 is specifically present in PRC1 complexes in lymphocytes but not in HSCs [70]. This suggests that the PRC1 complexes in HSCs have a unique composition that includes another Cbx member that can compensate for Cbx2. However, this particular Cbx protein has not yet been identified since studies on the role of Cbx4, Cbx6, Cbx7 and Cbx8 in HSC regulation are so far lacking.

HSC regulation by PRC2 members

In a 'genetical genomics' approach [81-82] followed by functional studies, the PRC2 member Ezh2 has been identified as being important in HSC regulation and aging (Figure 1). More than a decade ago, age-related properties of HSCs were found to be highly strain-dependent [83-84]. For example, HSCs from of DBA/2 mice (D2) mice are more abundant in number and have a higher turnover rate than C57BL/6 (B6) HSCs. Stem cell turnover rate was shown to be correlated with mean lifespan, and has therefore been considered as being one of the most important factors that underlies the aging process [85-86]. To uncover genes that are responsible for the differences in HSC frequency and turnover, gene expression profiling

of recombinant inbred strains from DBA/2 and C57Bl/6 was conducted [87-90]. Since 30 BXD strains were fully genotyped, the genetic basis of these differences in gene expression could be determined by quantitative trait loci (QTL) analysis [91]. Two genetic regions, one on chromosome 11 and one on chromosome 18, showed to be highly deterministic for the variation in cycling activity and HSC frequency, respectively [91].

Ezh2 was found to be transcriptionally controlled by the chromosome 18 locus [7] and HSCs of BXD mice that had inherited the B6 allele at the chromosome 18 locus showed significant higher expression levels of Ezh2 than mice that carried the D2 allele. Along hematopoietic differentiation, Ezh2 expression levels significantly decreased [7, 68] and enforced expression of Ezh2 prevented HSC exhaustion, as shown by repeated serial transplantations [7]. Whereas control HSCs cease to show long-term repopulating ability after three serial transplantations, the function of Ezh2 overexpressing stem cells was not impaired. Ezh2-deficient mice have no clear HSC phenotype, although B and T cell development and VDJ rearrangement showed to be affected [92, 93]. Likewise, inactivation of its homolog Ezh1 did not affect HSC function [92] [93]. In a recent study [94], the function of Ezh1 and Ezh2 was shown to be partially redundant with respect to self-renewal in embryonic stem cells. Upon loss of Ezh2, Ezh1 incorporated into PRC2 and compensated for its loss. This might also hold true for HSCs and might explain the lack of HSC phenotypes in knockout mice. However, several differences between Ezh1 and Ezh2 molecular functions have also been reported and these do not fully comply with functional redundancy [61].

In contrast to the effect of Ezh2 on HSCs, its binding partners Eed and Suz12 were shown to have negative effects on HSC and progenitors (Figure 1) [69, 95-96]. Eed loss of function using hypomorphic mice resulted in hyperproliferation of both primitive and more mature lymphoid and myeloid progenitors [69]. With age, the defects in proliferation capacity of progenitor cells in Eed mutant mice deteriorated and ultimately developed into leukemia [69]. Mutations in Suz12, which resulted in a truncated inactive form, enhanced HSC activity [95]. In another study, a sensitized $Mpl^{-/-}$ background was used to detect subtle changes in HSC function in mice heterozygous for individual PRC2 components ($Ezh2^{+/-}$, Eed $^{+/-}$ or $Suz12^{+/-}$). Ezh2, Eed and Suz12 were all found to restrict HSC activity [96]. The results for Ezh2 obtained in this study were not consistent with reports by Kamminga *et al*. [7], that were discussed above. Since both studies were performed in C57Bl/6 mice, the genetic background cannot account for this discrepancy. Yet, Ezh2 gene dosage and/or the balance between PRC2 complexes including either Ezh2 or its homolog Ezh1 might be important. Since we hypothesize that a strict balance in PRC complex compositions is central for HSC fate, we speculate that downregulation or overexpression of a PcG member does not necessarily have to result in strict opposite phenotypes. For example, upon overexpression of one particular PcG member (Cbx2), its family members (Cbx4, Cbx6, Cbx7 and Cbx8) will most likely be outcompeted

from the complex. In this case, the function of PRC complexes exclusively containing the overexpressed PcG member (Cbx2) will be studied. However, upon downregulation, the stoichiometry of PcG family members becomes disturbed and it will be difficult to foresee which homologs will take its position in the PRC complex and thus what exactly will be studied. Unfortunately, proteomic analysis regarding PRC complex compositions after PcG gene expression modification is generally lacking.

PcG target genes in HSC

In HSCs, the most important target identified for PcG genes thus far has been the tumor suppressor locus INK4a-ARF. This locus encodes two proteins, $p16^{Ink4a}$ and $p19^{Arf}$. p16 is a key regulator of cellular senescence since it functions as a cyclin-dependent-kinase-inhibitor and hampers cell cycle progression by activating the retinoblastoma (RB) pathway. p19 is important for p21/p53-mediated cell cycle arrest and apoptosis [97-98]. In $Bmi1^{-/-}$ mice, the INK4a-ARF locus showed to be responsible for the lymphoid and neurological defects, as a double deletion of Bmi1 and INK4a-ARF partially rescued the phenotype [99]. In mouse and human Bmi1-deficient HSCs, ineffective self-renewal has also been attributed to derepression of the INK4a-ARF locus and subsequent premature senescence [67, 72, 74]. p16, but not p19, was found to be a key-target of Ring1b in hematopoietic cells as well [78]. p16 was found to be upregulated in response to Ring1b deletion and INK4a-deficiency rescued the expansion defects of Ring1b-deficient lymphoid and myeloid precursor cells.

Chromatin immunoprecipitation experiments in mouse embryonic fibroblasts showed that INK4a-Arf is a critical target of many other PcG proteins as well [57, 100-102]. They repress its transcriptional activity by direct binding and catalyzation of H3K27 trimethylation. Ectopic expression of different PcG members in primary fibroblasts was shown to result in bypass of senescence and cellular immortalization [7, 57, 100-102].

Interestingly, several studies showed that p16 expression markedly increased with age in the hematopoietic and neuronal system and in the pancreas [103-105]. In the hematopoietic system, this increase was found to be restricted to the stem cell compartment (Lin^-, $Sca1^+$,$cKit^+$, $CD34^{low}$, $Flk2^{low}$) , because other bone marrow cells did not express p16 in an age-dependent fashion [103]. Using both p16-deficient and overexpression mice models, p16 was shown to promote aging by restricting stem cell self-renewal potential [103-105]. For instance, whereas normally the reconstitution ability of HSC declines with age, Janzen *et al*. [103] showed that this impairment could partially be mitigated by repressing p16. Thus, deficiency of a critical polycomb target gene, p16, showed to delay the age-associated decline of several adult stem cell types and therefore strongly argues for the involvement of PcG proteins in the aging process (Figure 2).

Nonetheless, critical INK4A-ARF-independent PcG targets in HSC also must exist, as the effect of both Eed and Suz12 downregulation showed to be INK4a-ARF independent [69, 95]. Hox genes such as HoxB4 and HoxA9, cell cycle-related genes such as p21, p27, cyclin D2, Geminin, and cdc6, C/EBPα target genes, and lineage developmental regulator genes (Pax5 and Ebf1) have all been suggested as being direct PcG targets in HSCs [66, 75, 78, 95, 96].

POLYCOMB GROUP PROTEINS AND THE DNA-DAMAGE RESPONSE

During each division of HSCs, either symmetrical or asymmetrical, replication of the genome results in copy errors. During evolution, several different DNA damage response mechanisms have developed that function as an initial step to repair these errors. However, during aging deficiencies in the DNA damage repair system have shown to limit the function of HSCs 106, 107. Appropriate regulation of DNA repair mechanisms is therefore crucial to maintain a proper HSC pool size with age, since extensive DNA damage is likely to trigger cellular senescence and apoptosis.

Very recently, several PcG proteins were identified to be involved in DNA damage responses in multiple cell types, including neural stem cells 108-111. In an attempt to identify factors that are specifically enriched in the chromatin-associated proteome after DNA damage, Chou *et al*. combined stable isotope labeling of cells in culture (SILAC), chromatin fractionation, and quantitative mass spectrometry and compared protein abundance of normal cells with cells damaged by UV-irradiation. Three polycomb group proteins, Suz12, Cbx8 and Ezh2, were found to be recruited to sites of damaged DNA. Depletion of these proteins caused cells to become more sensitive to ionizing radiation 108. In a different study, Bmi1 and Ring1b were found to be recruited to sites with double stranded breaks (DSB) 111 and contributed to the ubiquitinilation of γ-H2AX, an initial step in DSB repair. Bmi1 knockdown resulted in increased sensitivity to ionizing radiation and simultaneous depletion of Bmi1 and Ring1b resulted in an even severe accumulation of DNA damage after insult 111. Bmi1 also has shown to be involved in DSB repair in neuronal stem cells (NSC) 109. Overexpression of Bmi1 in these cells resulted in enhanced recruitment of ataxia-telangiectasia-mutated (ATM) kinase (ATM) to DSBs. ATM constitutes a crucial component of the DNA double-strand break (DSB) response machinery. Overexpression of Bmi1 subsequently resulted in resistance of NSC to radiation. Together, these results show a clear link between polycomb group proteins and the DNA damage response, and suggest yet another mechanism whereby PcG might safeguard genomic stability and cellular integrity during aging (Figure 2).

POLYCOMB GROUP PROTEINS, AGING, AND HEMATOLOGICAL MALIGNANCIES

The incidence of developing cancer increases exponentially with age [112]. The median age of diagnosis of acute myelogenous leukemia (AML) is 65 years [113] and fewer than 10% of the patients are children [114]. The observation of the age-distribution of cancer has been fundamental to the origin of the multi-stage theory of cancer [115]. This theory implies that most cancers arise from the accumulation of several mutations, and the probability of acquiring a sufficient number of detrimental mutations increases with time [112]. Because the lifespan of HSCs can surpass that of the organism [4, 116] and because of their potentially extensive replicative history, primitive hematopoietic cells are thought to be particularly subject to the accumulation of errors. However, according to the clonal succession theory, most cells in the HSC compartment are quiescent and only a few are activated at any given time [117]. In theory, dormancy lowers the risk of replication errors. This has recently been supported by advanced label-retaining studies [118].

Nevertheless, substantial evidence suggests that aging HSCs are likely targets for leukemic transformation. For example, it has been found that only a subfraction of cells, termed leukemic stem cells (LSC), possess the ability to initiate and sustain leukemia. Intriguingly, these LSCs express the same immunophenotypical markers ($CD34^+$, $CD38^-$) as normal human HSCs [119-121], which suggests that these rare LSCs originally derived from healthy HSCs. Second, the unique intrinsic property of stem cells to self-renew indefinitely is the foremost important criteria for malignant transformation, besides growth factor independency, differentiation blockage and escape from apoptosis. However, it has also been suggested that leukemias can arise from more committed progenitors that have acquired 'stemness' properties that allows them to self-renew infinitely. Yet, theoretically, malignant transformation of an HSC that already possesses self-renewal capacities requires fewer reprogramming events than transformation of a more differentiated cell without such self-renewal ability, and this is therefore more likely to occur in HSCs.

Cancer, including leukemias and lymphomas, has long been viewed purely as a genetic disease. Many patients with leukemia carry an abnormal karyotype as a consequence of chromosomal translocations. These translocations cause in-frame fusion of two otherwise separated genes. The resulting fusion product often disturbs proper regulation of proliferation, differentiation, growth factor signaling, senescence or apoptosis. Traditionally, these genetic abnormalities were considered to be the sole cause of malignant transformation of the cells. Typical examples of translocations frequently observed in AML patients are t(15;17) PML-RARa, t(8;21) AML1-ETO, Inv(16), CBFb-MYH11, t(7;11) NUP98-Hoxa9, and several varieties of 11q23 MLL rearrangements [122]. The 'Philadelphia Chromosome', the result of t(9;22) BCR-ABL fusion, is found in 95% of the patients with chronic myelogenous leukemia

(CML) which often used to progress into AML [123] but also in a subset of patients with acute lymphoblastic leukemia (ALL) [124]. However, it has been shown that some chromosomal translocations associated with hematopoietic malignancies can be present in healthy (aged) individuals, and do not seem to cause leukemia per se [125-128], arguing for cooperating events. An additional argument for the requirement of cooperating mutations for full malignant transformation is the observation that some oncogenic fusion proteins do not cause leukemia with 100% penetrance after cloning and expressing in murine hematopoietic cells, and leukemia develops only after many months [129, 130]. Together, these observations resulted in the (at least) two-hit model of leukemogenesis, stating that malignant transformation depends on both the activation of proto-oncogenes and deactivation of tumor-suppressor genes by genetic alterations [131]. Hence, cancer has been viewed as a disease driven by progressive genetic abnormalities, including chromosomal translocations and mutations in tumor suppressor genes (e.g. INK4a-ARF) and oncogenes.

However, about a decade ago it became apparent that cancer is also associated with profound epigenetic changes [132-134]. Tumor cells are associated with genome-wide DNA hypomethylation, gene specific hypermethylation, as well as genome-wide histone modifications [37, 135-138]. Whereas DNA hypomethylation results in chromosomal instability, both promoter hypermethylation and specific histone modifications represents an efficient mechanism for inactivation of tumor suppressor genes such as INK4a-ARF. Although it is now widely accepted that epigenetic alterations are common hallmarks of human cancer, epigenetic alterations are still viewed largely as a consequence of genetic lesions.

Yet, recently a critical role for polycomb (dys)regulation in the initial stages of tumorigenesis became apparent. First, it has been demonstrated that genes that are normally suppressed by polycomb complexes in stem cells are far more likely to undergo cancer-specific promoter DNA-hypermethylation than non-PcG target genes [139-141]. Half of the genes frequently hypermethylated were found to be premarked with Ezh2-containing PRC1 complexes that catalyzed the recruitment of DNA-methyl transferases (DNMT) in prostate and colon cancer, but not in healthy cells [140]. In a different study, Cbx7-containing PRC1 complexes were shown to be able to recruit DNMT to genes frequently found to be hypermethylated in cancer [142]. Next, in a study by Teschendorff *et al*., it was shown that aging contributes to this process. In aged blood cells and bone marrow-derived mesenchymal stem cells, silencing of PcG targets by promoter hypermethylation predisposed cells to become malignant [143].

Polycomb group genes are often deregulated in various types of cancer, including those of the hematopoietic system [144-145]. For example, Bmi1 overexpression is associated with both myeloid and lymphoid malignancies [146-149] and Cbx7 was expressed at elevated levels in human follicular lymphomas [150]. In turn, Ezh2 is overexpressed in several different

types of lymphomas [149, 151-153]. Very recently, a number Ezh2 mutations that inactivate its methyltransferase SET domain have been identified in different types of hematological malignancies, including in follicular and diffuse large B cell lymphomas [154] and in myeloproliferative syndromes [155, 156].

Taken together, we hypothesize that epigenetic dysregulation, for example by PcG misexpression, may precede genetic changes in premalignant progenitor or stem cells and set the stage for accumulation of additional genetic and epigenetic errors such as mutations, promoter hypermethylation and chromosomal instability that, together, may further drive tumor progression (Figure 2).

ADDITIONAL REMARKS AND FUTURE PROSPECTS

Polycomb group proteins are key factors in HSCs by regulating the transcriptional 'stemness' profile of the cell. Epigenetic changes imposed by PcG proteins can result in extensive effects on cell fate as they orchestrate DNA compaction and thereby affect expression of numerous genes. Traditionally, cellular aging and cancer, of which the incidence increases with age, have been viewed as genetic syndromes driven by the sequential accumulation of mutations. However, it is now clear that both hematopoietic stem cell aging and hematopoietic malignancies are accompanied by many epigenetic changes, including changes in PcG expression and function. This dysregulation can be held accountable for the disturbance of a variety of cellular processes, including senescence, DNA repair and cell fate decisions (self-renewal vs. differentiation), and it may contribute to aging as well as facilitating malignant transformation of hematopoietic cells. We therefore speculate that PcG proteins regulate the balance between aging (by limiting stem cell self-renewal) and the risk of developing cancer (excessive self-renewal) (Figure 2).

Whether dysregulation of PcG proteins in aged and malignant hematopoietic cells truly comprise the initial step of cellular aging and/or malignancy remains largely unknown, since at present there is no mechanistic evidence of a direct causal relationship. However, an intrinsic property in biology constitutes "epigenetic gambling" or "epigenetic drift". Subtle changes, that alter the epigenetic landscape of cells, constitute a mechanism to provide random changes and sufficient variation in cellular gene expression programs to ensure survival of the population (of cells or species) (Figure 2) [157]. Changes in PcG expression and its activity that occur during normal aging, are unlikely to uniformly affect all individuals. In addition, epigenetic modifications are thought to mediate, at least in part, the relationship between the environment and the genome. They could therefore provide a direct link between various external factors, such as radiation, exercise, and nutrition, which all have

been associated with healthy aging, and the risk of developing cancer (Figure 2). Further elucidation of the epigenetic background of hematopoietic stem cell aging and aging of other adult stem cell types could lead to strategies that stimulate healthy aging and prevent cancer development.

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Chapter 4

Polycomb Cbx family members mediate the balance between hematopoietic stem cell self-renewal and differentiation

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ABSTRACT

The balance between self-renewal and differentiation of adult stem cells is essential for tissue homeostasis. Here we show that in the hematopoietic system this process is governed by Polycomb Chromobox (Cbx) proteins. Cbx7 is specifically expressed in hematopoietic stem cells (HSCs), and its overexpression enhances self-renewal and induces leukemia. This effect is dependent on integration into Polycomb Repressive Complex-1 (PRC1) and requires H3K27me3 binding. In contrast, overexpression of Cbx2, Cbx4 or Cbx8 results in differentiation and exhaustion of HSCs. ChIP-sequencing analysis shows that Cbx7 and Cbx8 share most of their targets; we identified approximately 200 differential targets. Whereas genes targeted by Cbx8 are highly expressed in HSCs and become repressed in progenitors, Cbx7 targets show the opposite expression pattern. Thus, Cbx7 preserves HSC self-renewal by repressing progenitor-specific genes. Taken together, the presence of distinct Cbx proteins confers target selectivity to PRC1 and provides a molecular balance between self-renewal and differentiation of HSCs.

INTRODUCTION

Mature blood cells have a limited lifespan and are continuously replenished by HSCs, which have the potential to undergo both self-renewal and differentiation. Identifying the molecular basis of this process will be of great value for a fundamental understanding of normal and malignant hematopoiesis and holds promise for stem cell-based therapies in the future.

Proteins that maintain or modify the transcriptional profile of stem cells by organizing chromatin structure can be considered as key epigenetic candidates. Polycomb group (PcG) proteins were first discovered as important developmental regulators in *Drosophila* [1]. According to the classical PcG model, they assemble into at least two complexes, PRC1 and PRC2, which collaborate to repress gene transcription by catalysing histone modifications [2-3]. PRC2 is comprised of three core components (Ezh, Suz12, and Eed), of which the SET-domain containing Ezh protein contributes to gene repression by tri-methylating histone H3 on Lys 9 and 27 (H3K9me3, H3K27me3). PRC1 contains Pcgf (for example, Bmi1 and Mel18), Phc, Ring1 and Cbx, and catalyses mono-ubiquitylation of H2AK119.

Whereas in *Drosophila* each Polycomb component is encoded by a single gene, the number of genes encoding for PcG proteins has increased in mammals. However, the relevance of this diversification is unclear. Yet, expression patterns of PcG family members vary between distinct tissues and between various cell types within the same tissue [4-7]. For example, in the hematopoietic system, Bmi1 is specifically expressed in immature hematopoietic cells, while the expression of Bmi1 paralogue Mel18, is increased during differentiation [6]. This argues for the existence of cell-type and differentiation-stage specific PRC1 and PRC2 sub-complexes with unique molecular functions [8-11].

Analysis of the composition of the PRC1 complex showed that it contains a single representative of the Cbx family; either Cbx2, Cbx4, Cbx7 or Cbx8 [12-13]. The amino-terminal chromodomain of Cbx orthologs can bind H3K9me3 and H3K27me3 [14-15], albeit with unequal affinities [16-17]. Therefore, the Cbx proteins are key components for targeting PRC1 to specific genomic loci to regulate gene expression. Whereas several PcG proteins, such as Ezh2 and Bmi1, have shown to be important for HSC self-renewal [6, 18-22], no data are available for how different Cbx subunits contribute to HSC regulation.

By overexpressing individual Cbx family members in HSCs, we have interrogated the biological function of different Cbx-containing PRC1 complexes. We show that the decision of HSCs to self-renew or differentiate critically depends on the molecular composition of the PRC1 complex.

METHODS

Animals

10-16 week female C57BL/6 CD45.2 (Harlan, The Netherlands) or CD45.1 (bred at the Central Animal Facility) mice were used. All experiments are approved by the Animal Care Committee.

Cell purification

Long-term HSCs (Lin^-, $Sca1^+$, $c\text{-}Kit^+$, $CD48^-$, $CD34^-$ (BD) $CD150^+$ EPCR+), short-term HSCs (Lin^-, $Sca1^+$, $c\text{-}Kit^+$, $CD48^-$, $CD34^+$, $CD150^-$) and multipotent progenitor cells (Lin^-, $Sca1^+$, $c\text{-}Kit^+$, $CD150^-$, $CD34^+$) were sorted from pooled BM cells as described previously [23].

Common lymphoid progenitors (Lin^-, $CD127^+$, $c\text{-}Kit^{mid}$, Sca^{mid}), common myeloid progenitors (Lin^-, $CD127^-$, $c\text{-}Kit^+$, $Sca1^-$, $CD16/32^{mid}$, $CD34^{mid}$) , granulocyte-macrophage progenitors (Lin^-, $CD127^-$, $c\text{-}Kit^+$, $Sca1^-$, $CD16/32^{high}$, $CD34^{high}$) and megakaryocyte-erythroid progenitors (Lin^-, $CD127^-$, $c\text{-}Kit^+$, $Sca1^-$, $CD16/32^{low}$, $CD34^{low}$) were isolated by staining with Alexa-Fluor-conjugated lineage antibodies (B220 clone RA3-6B2, Biolegend 135010, 1:500, Gr1 clone RB6-8C5, Biolegend 108422, 1:75, Mac1 clone M1/70, Biolegend 101222, 1:100, Ter119 clone TER119, Biolegend 116220, 1:100, CD3 clone 17A2, Biolegend 100216, 1:150), CD127-PE (clone A7R34, Biolegend 135010, 1:250), Sca1-Pacific Blue (clone D7, Biolegend 108120, 1:150), cKit-APC (clone 2B8, BD Biosciences 561074, 1:75), CD16/32-PeCy7 (clone 93, eBioscience 25-0161-82, 1:75) and CD34-FITC (clone RAM34, BD BD Biosciences, 1:100). Sorting gates were set according to Challen *et al*. [24]. Granulocytes ($Gr1^+$), T-cells ($CD3\varepsilon^+$) and B-cells ($B220^+$) were isolated using B220-Pacific blue (clone RA3-6B2, Biolegend 103227, 1:1000), CD3ε-APC (clone 145-2C11, eBiosciences 17-0031, 1:100) and Ly-6G(Gr1)-AlexaFluor700 (1:100).

Retroviral vectors

Retroviral SF91-IRES-GFP vector (gift of C. Baum, Hannover Medical School, Germany) was used as backbone for cloning cDNAs upstream of IRES. Cbx2, Cbx4, and Cbx7 cDNA clones were ordered from OpenBiosystems (accession numbers: Cbx2 BC035199, Cbx4 BC117801, Cbx7 BC021398). myc-flag-Cbx8 was cloned from PINCO [42] into SF91.

N-terminal flag-tagged Cbx7 was generated by PCR using forward primer containing flag-sequence F:5'GCAGCGGCCGCATGGACTACAAGGACGACGATGACAAGATGGAGCTGTCAGCCATAG GCGAGCAGGTG-3' and reverse R:5'-GGTCGACAGGGGAAGCCGCTATTCACAG-3', and cloned into TOPO-blunt. Cbx7 chromodomain mutant ($Cbx7^{AA}$: K31W32 to A31A32) was cloned via PCR on TOPO-flag-Cbx7 using F:5'-CAAAGTTGAATATCTGGTGGCGGCGAAGGATGGCCCCC-CA-3' and R:5'-CTTGGGGGGCCACCTTTCGCCGCCACCAGATATTCAACTT-3'.

Cbx7$^{\Delta Pc}$ (ΔTVTFREAQAAEGF) was cloned by insertion of Xba1 restriction sites flanking the Pc-box using F:5'-GCTCTAGATTCCGAGACCGCAACGAGAAG-3' and R:5'-GCTCTAGAGACG-GAGTTGGCGGTGATG-3'. The Pc-box was then deleted by Xba1 digestion and self-ligation. Subcloning from TOPO into SF91 was performed by Not1-Sal1 digestion and ligation. All constructs were verified by Sanger sequencing.

Retroviral overexpression in primary BM

Post-5FU primary BM cells from female C57BL/6 (CD45.2) were isolated from tibia, femora and pelvic bones and transduced with indicated vectors as described [43]. EGFP+ cells were sorted using a MoFlo flowcytometer (BeckmanCoulter).

Downregulation of Cbx7 in primary BM

Lentiviral construct containing a short hairpin against Cbx7 (CCGGCCTCAAGTGAAGTTACCGTGACTCGAGTCACGGTAACTTCACTTGAGGTTTTTG [45]) was generated by cloning annealed oligos into the AgeI and EcoRI cloning sites of pLKO.1 (Addgene plasmid 10878 [44]). As a control, a pLKO.1 vector containing a scrambled RNAi (CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG [46]) was used (Addgene plasmid 1864).

20% confluent 293T cells (T75) were transfected with 3µg of pLKO1-shRNA (Cbx7 or scrambled), 0.7µg of pCMV-VSV-G, and 0.3µg of pCMV-8.91 plasmids in DMEM . After 18h medium was changed to StemSpan. Virus supernatant was added to HSPCs 18h later. Cells were selected with 3µg/mL puromycin 48h after infection. 72h later cells were plated in assays. Liquid cultures were maintained under selective antibiotics.

In-vitro assays

Post-5FU GFP+ HSPCs cells were collected in StemSpan (StemCell Technologies) supplemented with 10% FBS, 300 ng/mL rSCF (Amgen), 20 ng/mL rmIL11 (R&D Systems), 1 ng/mL Flt3 ligand (Amgen), and penicillin and streptomycin. Cytospots were generated by spinning at 800rpm in 200µL FCS (Shandon Cytospin3) 10-days after culturing. 10-day cultured cells were lysed, fixed and permeabilized according to manufacturer's protocol (BD™ Phosflow, BD Biosciences) and stained with 1.2 µg/mL DAPI. Apoptosis was assessed by staining 10-day cultured cells with 10µg/mL 7AAD (Sigma) and 50µL/mL Annexin-V (IQ-Products) in calciumbuffer. Cells were analyzed by flowcytometry (LSRII, BD Biosciences).

Post-5FU transduced 7-day cultured GFP+ cells were plated in methylcellulose supplemented with 100ng/mL mSCF and 20ng/mL rmGM-CSF (R&D systems). After a 6-day incubation period, CFU-GM colonies were scored. Cells were collected by scraping and washed 3x in PBS. All cells were collected in fresh methylcellulose medium, vortexed and replated. For

single colony replating, 50 single colonies were picked, made single-cell by vortexing in new methylcellulose and plated in a 35mm-dish. Colonies were scored 7 days later.

Cobblestone-area forming cell (CAFC) assays were performed as previously described [47]. Directly after transduction, GFP+ cells were plated in limiting dilution on a FBMD stromal cell layer and CAFCs were scored weekly.

BM transplantation

4.5-7.5x10^6 freshly transduced HSPCs (CD45.1) were transplanted into lethally irradiated (9.0Gy) CD45.2 mice, without prior GFP sorting. Transduction percentages were 20%-40%. The relative contribution of transduced cells (GFP+) compared to identical treated non-transduced cells (GFP-) was determined by blood withdrawal from the retro-orbital plexus. Red cells were subjected to lysis and white blood cells were stained with CD45.1-PE (Clone A20, Biolegend 110708, 1:200), B220-Pacific blue (clone RA3-6B2, Biolegend 103227, 1:1000), CD3ε-APC (clone 145-2C11, eBiosciences 17-0031, 1:100) , Ter119-Pe/Cy7 (Clone TER-119, Biolegend 116222, 1:200), CD11b-AlexaFluor®700 (Clone M1/70, Biolegend 101222, 1:100), and Ly-6G(Gr1)-AlexaFluor®700 (clone RB6-8C5, Biolegend 108422, 1:100).

At clear signs of morbidity, blood was taken from the orbital plexus. The mouse was sacrificed and BM cells were isolated by crushing. Single cell suspensions were generated from different organs (spleen, lymph nodes, liver, thymus). Cell numbers were counted. Bloodsmears and cytospins were made and stained by MGG. Immunophenotypic analysis was performed as above. In a few cases, spleen cells were additionally stained with CD3-AF700 (clone 17A2, Biolegend 100216, 1:150), CD4-APC (clone RM4-5, Biolegend 100516, 1:200), and CD127-PE (clone A7R34, Biolegend 135010, 1:250), or CD41-PE (clone MWReg30, Biolegend 133906, 1:100) and CD16/32-PeCy7 (clone 93, eBioscience 25-0161-82, 1:75) .

Transduction and transplantation of purified hematopoietic cells

LTHSCs, STHSCs, MPPs, and myeloid progenitors were FACS purified as explained above, and pre-stimulated for 24h in StemSpan with supplements. CLPs were pre-stimulated in StemSpan supplemented with 10% FBS, 100ng/mL SCF, 50ng/mL Flt3, 5ng/mL IL7 and P/S. 24h after pre-stimulation cells were transferred to retronectin-coated wells and virus-supernatant produced in StemSpan was added. GFP+ cells were sorted 24h later.

Single GFP+ cells were seeded into liquid cultures as described in [23]. 14 days later, wells were scored for the presence of a clone and the clone size was assessed. Colony size-1 represents colonies containing 1-30 cells, 2 31-100 cells, 3 101-1000 cells, 4 ~5000 cells, 5 ~15000 cells, and size 6 >50000 cells. Purified subpopulations were also plated in CAFC-assay (1, 3, 10 cells/well).

750 transduced CD45.1+ GFP+ cells were transplanted in CD45.2 recipients plus 0.75x10^6 CD45.2+ fresh BM cells. 12 months later BM from mice that showed >1% chimerism was analyzed.

Immunoprecipitation

Immunprecipitation using M2 Flag-agarose beads (Sigma) were done according to standard protocol in IP150 buffer (50 mM Tris-HCl at pH7.5, 150 mM NaCl, 5% glycerol, 0.2% Igepal, protein inhibitor cocktail (Roche)) in a myeloid celline (32D).

Chromatin-immunoprecipitation assays (ChIP) of transduced HSPCs were in essence performed as described [48]. Flag-ChIP using M2 Flag-agarose beads was performed in IP150 buffer and complexes were washed 4x in 500mM wash buffer (1% Triton X-100, 0.1% SDS, 500mM NaCl, 2mM EDTA pH8.0, 20mM Tris-HCl pH8.0) and the final wash step was performed in TE buffer. All other ChIPs were performed in IP buffer (66.7mM Tris-HCl, 100mM NaCl, 5mM EDTA, 0.2% NaN3, 1.67% Triton-X-100, 0.33% SDS) and complexes were washed in 500mM wash buffer followed by LiCl buffer (20mM Tris-HCl pH8.0, 1mM EDTA, 250mM LiCl, 0.5% NP40, 0.5% Na-deoxycholate) and a final wash step in TE. 1ug of rabbit-anti-CBX8 (LAST) [49], rabbit-anti-Cbx7 (Millipore, 07-981) and rabbit-anti-H3K27me3 (Upstate, 07-449) or IgG from rabbit serum (Sigma, I8140) were used per IP. Prot A and Prot G fast-flow sepharose (GE Healthcare) were used for pull down. Primer sequences are available upon request.

Western blotting

SDS-PAGE was performed according to standard protocols. Membranes were incubated with rabbit-anti-Cbx2 (1:1000, Abcam, ab80044), rabbit-anti-CBX8 (1:1000, LAST), rabbit-anti-Cbx7 (1:100, Santa Cruz, P-15), rabbit-anti-Ring1b (1:1000, NAST) [50], goat-anti-Mel18 (1:250, Abcam, ab5267), mouse-anti-Bmi1 [51] (1:1000, AF27), mouse-anti-H3 (Cell Signaling 3638, 1:300), rabbit-anti-actin (Cell Signaling 4970, 1:5000)

ChIP-sequencing

DNA samples obtained from the ChIP assays were adaptor-ligated and amplified (NEB, E6040) and analyzed by Illumina Genome Analyzer II. Duplicate reads were removed to avoid PCR bias. Using Bowtie alignment algorithm for Illumina sequence format (version 1.1.2) on the Galaxy server [52-54], the sequence datasets were mapped to the mouse genome (NCBI37/mm9). Peak detection was performed using MACS1.0.1 [55-56] (Genome size 1.87e+9, tag size 36bp, cutoff peak detection 1e-5). Chromosomal positions were annotated to the RefSeq/ENSEMBL database. To define the distribution of peaks across the genome, we used Bioconductor (version 2.9) package ChIPpeakAnno [57] and the build in data set “TSS.mouse.NCBI37”. For other features a custom annotation set was created by BioConductor/R package BiomaRt [58].

Enriched genomic regions for Cbx7 and Cbx8 were determined using the empty vector (control) ChIP-signal as negative control. In addition, a cross comparison was performed were Cbx7 signal served as control for Cbx8 peak calling, and the other way around. This comparative analysis determines regions where the ChIP signals are enriched relative to control ChIP (empty vector), or to the alternative Cbx protein, by a conditional binomial model (75-bp window, 0.1 FDR cut-off). All statistics and plotting were performed in *R*.

Gene network analysis

Gene expression network analysis of Cbx7 and Cbx8 targets was performed as described previously [33] using gene expression data of 4 primary cell subsets (LSK, L-S-K+, Gr1+, Ter119+). Probes were defined as differentially expressed during differentiation if (Vmax-Vmin)/stdev≥4 ($p<0.05$), assuming normal distribution (3σ). Only transcripts that show significant differential expression were subjected to relevance network analysis as described by Voy *et al*. [32]. Pearson correlation (r) between each pair of transcripts was measured and transcripts with -0.8<r>0.8 were marked as connected. Networks were visualized in Gephi software (version 0.8beta) [59] and spatially arranged by built-in Yifan-Hu and Fruchterman-Reingold algorithms. A build-in modularity function was used to partition the network into significant tight connected communities ('modules'). Significant differential clustering between Cbx7 and Cbx8 targets was tested using binomial distribution.

Statistical analysis

Statistical significant differences of growth curves was assessed using 'compareGrowthCurves' of the statmod software package *(R)*. CAFCs by limiting dilution were calculated by maximum likelyhood as described in [60]. Other statistical analyses were performed by two-tailed t-tests assuming equal variances by SPSS or Excel.

RESULTS

Distinct expression of Cbx family members during hematopoietic differentiation

First, we assessed the expression of Cbx family members in purified hematopoietic subpopulations. Cbx7 is highly expressed in long-term HSCs (LT-HSCs), and its transcript levels gradually decreases upon lineage commitment (Fig 1a,b). However, Cbx7 is also highly expressed in differentiated lymphoid lineages (Fig 1a, Fig S2). The Cbx8 transcript is equally expressed in various primitive hematopoietic cells. However, at the protein level, Cbx8 levels increase on lineage commitment, indicating a possible role for post-transcriptional regulation [10, 23] . Cbx4 is abundant in most hematopoietic cell populations while Cbx2 is rather marginally but ubiquitously expressed (Fig 1a,b, Fig S2). Notably, of all Cbx family members, the Cbx7 transcript is most abundantly expressed in LT-HSCs. These data suggest that the PRC1 complex in HSCs preferentially contains Cbx7.

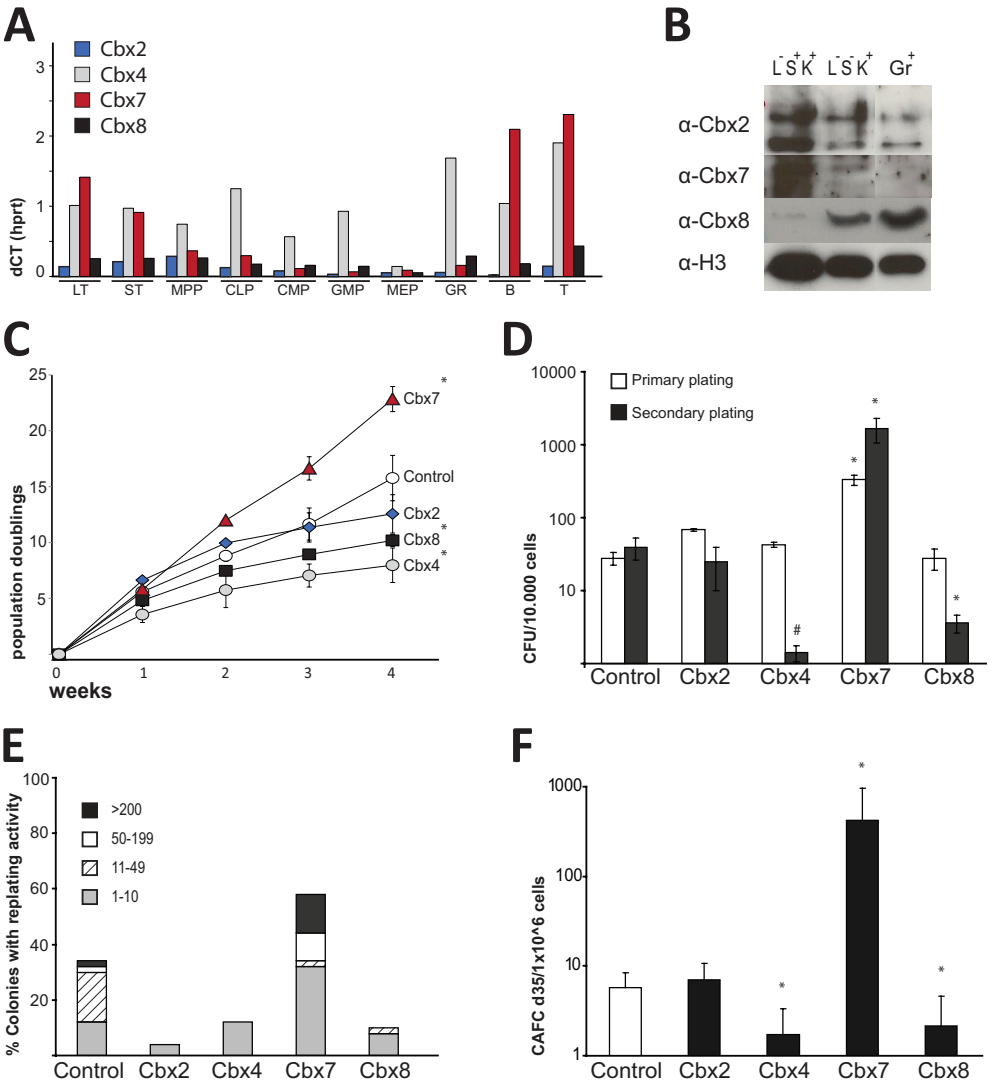


Figure 1. Enforced expression of Cbx genes reveals distinct effects on *in vitro* progenitor and HSC potential. A Gene expression of Cbx family members in different hematopoietic cell populations. Bars represent mean of 2-3 technical replicates. See Table S7 for raw data. **B** Protein abundance of Cbx2, Cbx7 and Cbx8 in Lin- Sca1+ cKit+ (LSK), Lin- Sca- cKit+ (prog), and Gr1+ cells. **C** Mean number of population doublings (± s.e.m.) for HSPCs overexpressing either control (n=4), Cbx2 (n=3), Cbx4 (n=3), Cbx7 (n=4) or Cbx8 (n=4). *n* represents independent experiments, *p≤0.05. See Table S7 for raw data. **D** Mean colony-forming ability (±s.e.m) of Control and Cbx-overexpressed HSPCs (CFU=colony forming unit, Control n=15, Cbx2 n=6, Cbx4 n=6, Cbx7 n=18, Cbx8 n=13. *n* represents independent experiments. t-test *p<0.05, #p=0.06). **E** Secondary colony formation upon replation of individual primary colonies (n=50 primary replated colonies per sample) **F** Mean day-35 CAFC frequencies (±s.d.) of Control and Cbx-overexpressed HSPCs (Control (n=10), Cbx2 (n=6), Cbx4 (n=6), Cbx7 (n=13) and Cbx8 (n=8). *n* represents independent experiments. t-test *p<0.05. Uncropped images of blots are shown in Supplementary Figure 9a.

Cbx family members affect *in vitro* self-renewal potential

Since Cbx orthologues compete for integration into PRC1 [12-13], overexpression of individual Cbx family members can change the composition of PRC1 complexes in favour of inclusion of the overexpressed Cbx protein. We overexpressed individual Cbx family members in post-5FU-treated bone marrow (hereafter referred to as hematopoietic stem and progenitors cells (HSPCs)). In cytokine-driven cultures, Cbx7 overexpression results in a strong proliferative advantage (Fig 1c) and substantially increases the fraction of cells in the S-G2/M phase, without affecting apoptosis (Fig S3a,b). In contrast, Cbx4 or Cbx8 overexpression leads to decreased proliferation (Fig 1c, Fig S3a,b), while Cbx2 overexpression does not lead to any detectable changes in proliferation (Fig 1c). In liquid cultures, Cbx7 overexpressing HSPCs retain an immature blast-like morphology (Fig S3c), whereas the majority of Cbx8 cells differentiate into myeloid cells within 10 days (Fig S3c).

To measure self-renewal, cells were plated in methylcellulose-based colony assays, followed by whole-dish or single colony replating. Overexpression of Cbx7 substantially increases the colony-forming ability. This effect is even more pronounced after replating (Fig 1d). Most single Cbx7 colonies have a high replating potential; 15% of the primary colonies can form more than 200 secondary colonies (Fig 1e). In contrast, overexpression of Cbx2, Cbx4, and Cbx8 results in lower numbers of secondary colonies (Fig 1d,e), indicating diminished self-renewal.

The effect of Cbx overexpression was further studied in the cobblestone-area-forming-cell (CAFC) assay (Fig 1f). Strikingly, Cbx7 overexpression leads to a strong increase (~100-fold) in day-35 CAFC activity, which reflects the activity of the most primitive hematopoietic cells. In contrast, overexpression of Cbx4 or Cbx8 results in significantly reduced number of CAFCs.

In summary, overexpression of individual Cbx proteins causes distinct effects on self-renewal *in vitro*. Cbx7 is unique among the Cbx members in its ability to stimulate self-renewal *in vitro*.

Cbx family members affect *in vivo* HSC repopulating potential

Next, we tested the repopulating ability of HSPCs overexpressing Cbx2, Cbx4, Cbx7 or Cbx8 in a competitive transplantation setting. Mice transplanted with Cbx2-overexpressing HSPCs only showed B-cell reconstitution (Fig S4a), suggesting that Cbx2 has a role in lymphopoiesis, but not myelopoiesis. However, HSPCs that overexpress Cbx2, Cbx4 or Cbx8 all fail to contribute to long-term hematopoietic reconstitution (Fig 2a), suggesting a rapid exhaustion of progenitors and HSCs. In contrast, cells that overexpress Cbx7 have a prominent competitive advantage over non-transduced cells, as revealed by high chimerism levels in peripheral blood (Fig 2a).

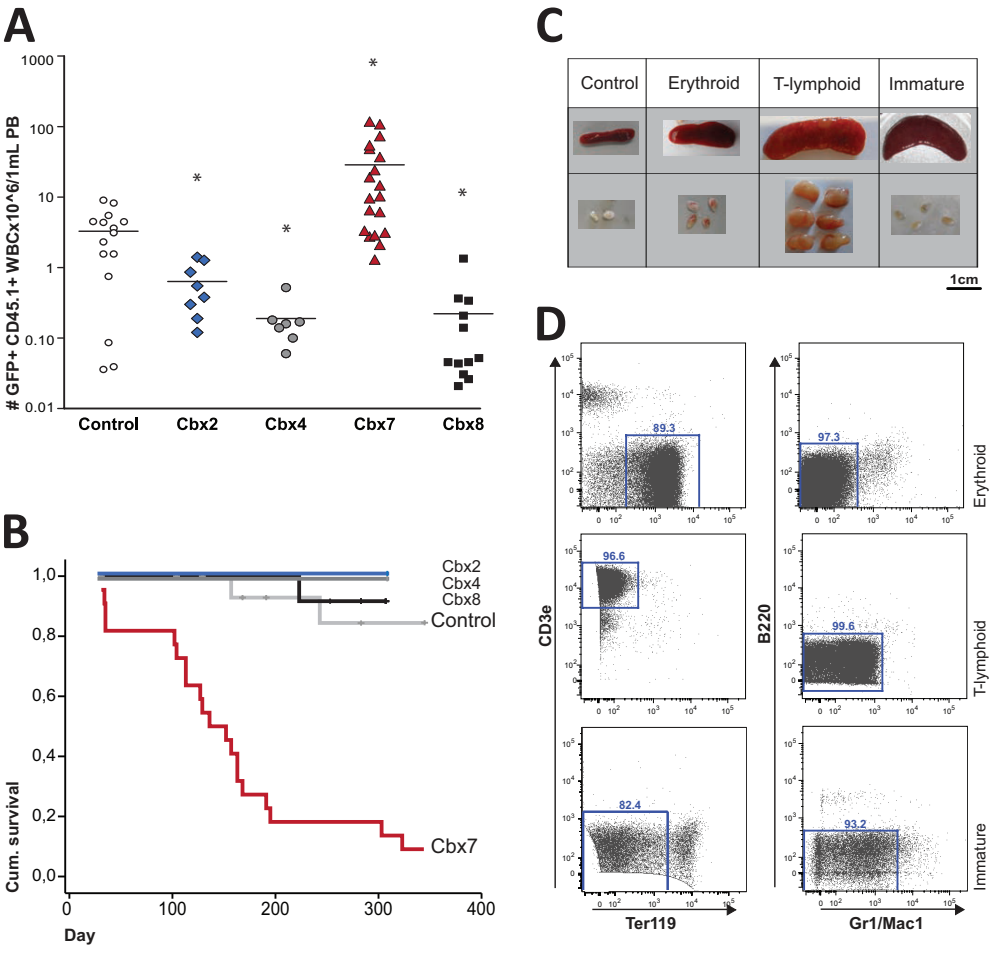


Figure 2. Cbx7 induces distinct types of leukemia, whereas Cbx2, Cbx4 and Cbx8 induce a competitive disadvantage *in vivo*. Post-5FU treated bone marrow cells from CD45.1 mice were isolated and transduced with Control or Cbx-overexpressing retroviral vectors. 4.5-7.5x10^6 freshly transduced cells were then transplanted into lethally irradiated CD45.2 mice, without prior sorting for GFP. **A** Post-transplantation chimerism levels of individual mice as measured by the absolute number of GFP$^+$ CD45.1$^+$ donor cells in peripheral blood at week 16 (control n=17, Cbx2 n=8, Cbx4 n=7, Cbx8 n=12, or at the day of sacrifice (Cbx7 n=22). Line represents average chimerism levels. t-test *p<0.05). **B** Kaplan-Meier survival curve for mice transplanted with control, Cbx2, Cbx4, Cbx7 or Cbx8 transduced bone marrow cells (control n=17, Cbx2 n=8, Cbx4 n=7, Cbx7 n=22, Cbx8 n=12). **C** Spleen and lymph nodes from control, and Cbx7-induced leukemic mice. **D** Representative FACS plots of GFP$^+$ spleen cells showing distinct dominant cell populations in Cbx7-induced leukemias. Data from two independent transplantation experiment are combined. Gr1/Mac1: mixture of Gr1 and Mac1 antibodies with the same fluorochrome.

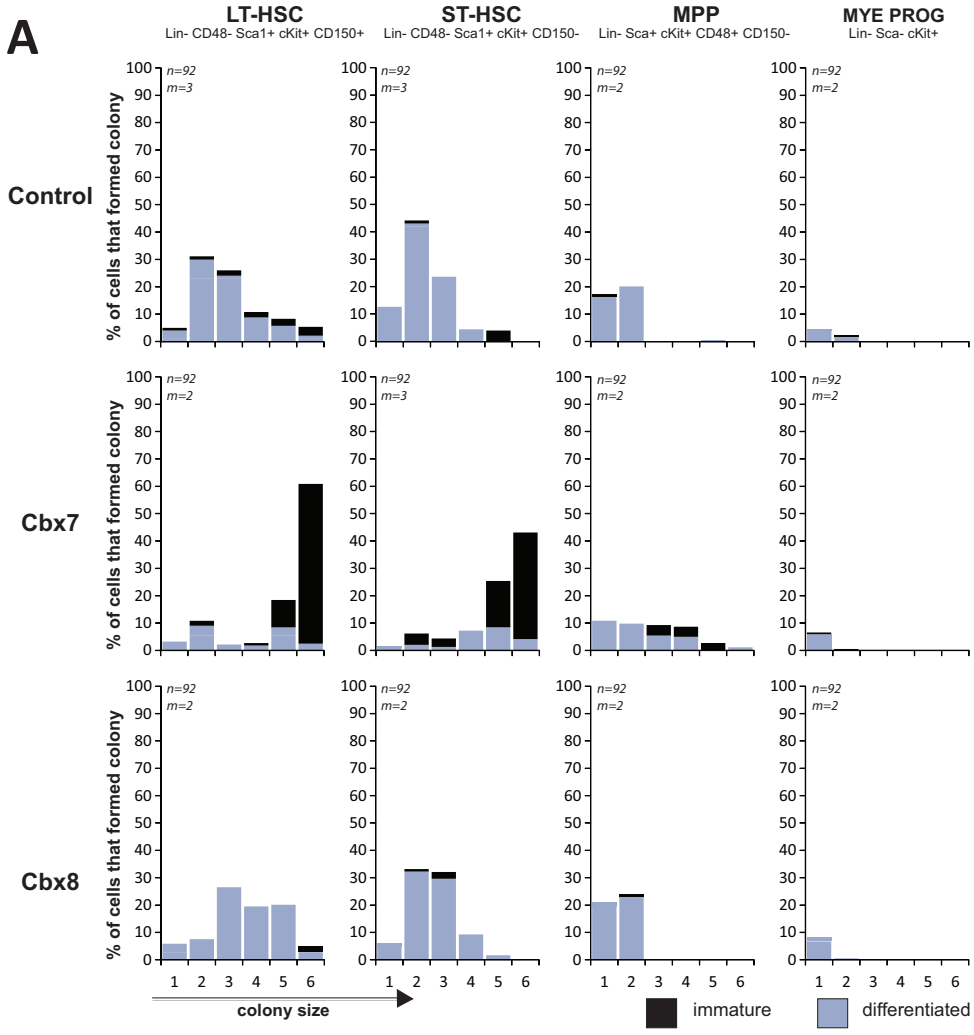
Out of 22 mice, 20 reconstituted with Cbx7-overexpressing HSPCs developed lethal leukemias with variable latencies (Fig 2b-d). Twelve mice were diagnosed with T-cell leukemia. These mice showed increased white blood cell (WBC) counts (Fig S4d, Table S1), enlarged lymph nodes and splenomegaly (Fig 2c, Fig S4d). Thymus and liver were variably affected (Table S1). Cells were CD3+ (Fig 2d), CD4+, and CD127+, (Fig S4b, Table S1). Interestingly, Cbx7 protein was found to be elevated in human lymphoid tumors 24. Previously, overexpression of Cbx7 in collaboration with c-Myc, in the lymphoid compartment has been shown to cause T-cell lymphomas 24. Here, we neither observed upregulation of c-Myc nor of other major oncogenes involved in lymphomagenesis (Fig S5).

The second type of leukemia developed with a short latency (~4 weeks for 3 out of 4 mice) and was classified as an immature leukemia. Malignant cells did not express any of the lineage markers used for analysis (Fig 2d, Fig S4c, Table S1). Peripheral WBC counts were increased (Fig S4d, Table S1) and cells showed a blast-like morphology (Fig S4d). Finally, 2 out of 22 mice developed erythroid leukemias. Spleens were mildly enlarged and one mouse showed hemorrhagic lesions in lymph nodes (Fig 2c). Peripheral WBCs and erythrocytes were decreased (Fig S4d, Table S1), whereas the number of reticulocytes was increased (Fig S4d). In addition, bone marrow and spleen samples revealed numerous erythroid precursors at variable stages of maturation (Fig S4d). Malignant GFP+ cells expressed Ter119 (Fig 2d, Table S1). For two mice it was not possible to determine the leukemia subtype (mice number 2 and 20 in Supplementary Table S1).

These results show that Cbx7 has a strong oncogenic potential *in vivo*, whereas Cbx2, Cbx4 and Cbx8 all induce HSC exhaustion.

Cbx7 induces self-renewal in multipotent cells, but not in restricted progenitors

As post-5FU bone marrow contains a heterogeneous pool of primitive cells, we investigated which specific cell population is responsible for Cbx7-induced self-renewal. We overexpressed Cbx7 and Cbx8 in purified LT-HSCs, short-term HSCs (ST-HSCs), multipotent progenitors (MPPs), or myeloid progenitors, and compared their effect on the proliferative response in these purified populations at the clonal level. Single transduced cells were seeded in stromal-free cytokine-supplemented cultures, and colony-forming ability was scored 14 days later (Fig 3a). Overexpression of Cbx7 enhanced the proliferative capacity of LT-HSCs, ST-HSCs and MPPs, but not of myeloid progenitors (Fig 3a). Morphological analysis (Fig 3b) showed that many of the Cbx7 colonies consisted of undifferentiated cells. In contrast, Cbx8 induced differentiation of LT-HSCs and ST-HSCs, because only a few colonies consisted of immature cells.


A
LT-HSC
Lin- CD48- Sca1+ cKit+ CD150+
ST-HSC
Lin- CD48- Sca1+ cKit+ CD150-
MPP
Lin- Sca+ cKit+ CD48+ CD150-
MYE PROG
Lin- Sca- cKit+
Control
Cbx7
Cbx8
% of cells that formed colony
n=92
m=3
m=2
colony size
immature
differentiated

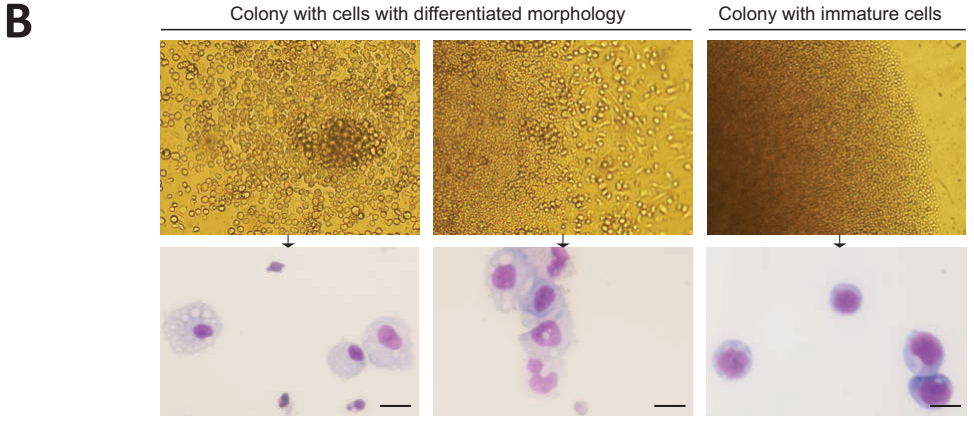

B
Colony with cells with differentiated morphology
Colony with immature cells

Figure 3. Cbx7 increases the proliferative capacity of purified multipotent hematopoietic subsets.
A Clonal analysis of the proliferative capacity of indicated purified hematopoietic cells transduced with Control, Cbx7 or Cbx8 retrovirus. Single transduced cells were seeded in cytokine rich media and the colony size that these cells could produce was scored 14 days later. The figure represents the variability in colony forming ability of single cells, scoring 92 cells (n) per independent experiment. Two or three independent experiments (m) performed per sample. **B** The cell type of which the colonies consisted at day 14 was assessed using microscopical analysis of the colony itself and MGG-staining of cytospins from cells of these colonies. Scale bar, 10μM.

In the FBMD-stromal co-cultures, day35+ CAFC activity is restricted entirely to the most primitive LSK $CD48^-$ $CD150^+$ cells [25-26]. (Fig 4a). However, Cbx7 overexpression induced late cobblestone-forming activity also in ST-HSCs and MPPs, but not in myeloid progenitors (Fig 4a). Cbx8 overexpression in ST-HSC and MPPs fails to induce such an effect.

We transplanted 750 Cbx7-overexpressing LT-HSCs in lethally irradiated recipients. Bone marrow analysis 12 months post-transplantation shows a significant increase in the frequency of GFP^+ ST-HSCs and MPPs, while the absolute number of lineage restricted progenitors (common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs)) and fully differentiated cells is reduced (Fig 4b). In contrast, when Cbx8 is overexpressed, lower numbers of all GFP^+ hematopoietic cell subsets are observed, which indicates exhaustion of progenitors and HSCs *in vivo* (Fig 4b). Importantly, when Cbx7 is overexpressed in myeloid progenitors or lymphoid progenitors, these cells do not contribute to hematopoietic reconstitution (Fig 4c).

Collectively, these results suggests that Cbx7 overexpression induces self-renewal of LT-HSCs, ST-HSCs and MPPs, and restricts their differentiation.

Loss of Cbx7 function reduces HSC self-renewal

To address whether the activity of HSCs and progenitors is dependent on Cbx7, we downregulated Cbx7 in HSPCs (Fig 5a). Knockdown of Cbx7 results in a proliferative disadvantage in liquid culture (Fig 5b), a reduction in their colony forming ability (Fig 5c), and a reduced frequency of CAFCs (data not shown). Cbx7 is therefore required for HSC and progenitor activity *in vitro* and other Cbx family members cannot compensate for its loss.

Next, we assessed whether the effect of Cbx7 overexpression on HSCs is dependent on its integration into PRC1. A mutant Cbx7 allele was generated in which the Pc-box was deleted [27]. This abrogates binding to Ring1b and Bmi1 (Fig S6b), and integration into PRC1 can therefore not occur. However, this mutant is still able to bind chromatin, although less efficiently (Fig S6c). In contrast to $Cbx7^{WT}$, overexpression of Cbx7ΔPc in HSPCs neither results in increased proliferative capacity in liquid culture (Fig S6a), nor to increased self-renewal as measured by the number of colony forming units (CFUs) (Fig 6a). Furthermore, $Cbx7^{\Delta Pc}$ does

not lead to an increased frequency of day-35 CAFC activity (Fig 6b) and transplanted Cbx7$^{\Delta Pc}$ HSPCs fail to contribute to hematopoietic regeneration (Fig 6c). We reason that Cbx7$^{\Delta Pc}$ acts in a dominant-negative manner by binding to target loci independently of PRC1, thereby blocking binding of endogenous PRC1 complexes.

Cbx proteins target PRC1 to chromatin by their chromodomain which recognizes H3K9/27me3 [14-17]. To study whether Cbx7-induced self-renewal is dependent on recognizing this histone mark, a chromodomain-mutant (Cbx7^{AA}) was generated [27]. This amino-acid substitution abrogates binding to chromatin (Fig S6c), but it retains the ability to bind PRC1 components Bmi1 and Ring1b (Fig S6b). It therefore competes with endogenous Cbx proteins. Cbx7^{AA}, like Cbx7$^{\Delta Pc}$, acts in a dominant-negative manner because its overexpression results in a severe impairment of *in vitro* clonogenicity (Fig 6a), self-renewal (Fig 6b), as well as *in vivo* repopulating ability (Fig 6c).

We verified whether perturbations of Cbx gene expression change the molecular composition of the PRC1 complex. Using Flag immunoprecipitation, we show that the core PRC1 component, Ring1b, interacts with both overexpressed Flag-Cbx7 and Flag-Cbx8 (Fig 6d). Both Pcgf family members, Bmi1 and Mel18, bind equally to Cbx7 and Cbx8 (Fig 4d), suggesting equal capabilities of overexpressed Cbx7 and Cbx8 to integrate into endogenous PRC1. As expected, binding between Cbx7 and Cbx8 was not detected. (Fig 6d). In addition, chromatin immunoprecipitation (ChIP) data show dissociation of endogenous Cbx8 from the *Ink4b-Ink4a-Arf* locus upon overexpression of Cbx7, and vice versa (Fig S7a-b).

These data support the notion that Cbx7 is required for self-renewal and that the contrasting phenotypes after overexpression of Cbx proteins are caused by compositional rearrangements of PRC1.

Cbx7 and Cbx8 functional targets

As Cbx7 was the only Cbx family member inducing self-renewal, we hypothesized that Cbx7 and Cbx8 bind to different genomic loci. Cbx8 was selected because its protein expression is reciprocal to Cbx7 and its overexpression showed a striking contrasting phenotype.

We evaluated whether Cbx7 and Cbx8 affect Ink4b-Ink4a-Arf, because studies have implicated that phenotypes of Bmi1 are, partially, dependent on this locus [28-29]. We show that both Cbx7 and Cbx8 bind throughout this locus (Fig 7a,b, Fig S7a,b,d) which is enriched for H3K27me3 as evidenced by ChIP experiments (Fig S7c-d). No changes in expression of p15, p16 and p19 were observed (Fig S7e). Therefore, the contrasting HSC phenotypes induced by Cbx7 and Cbx8 are not explained by differential capabilities to repress this locus.

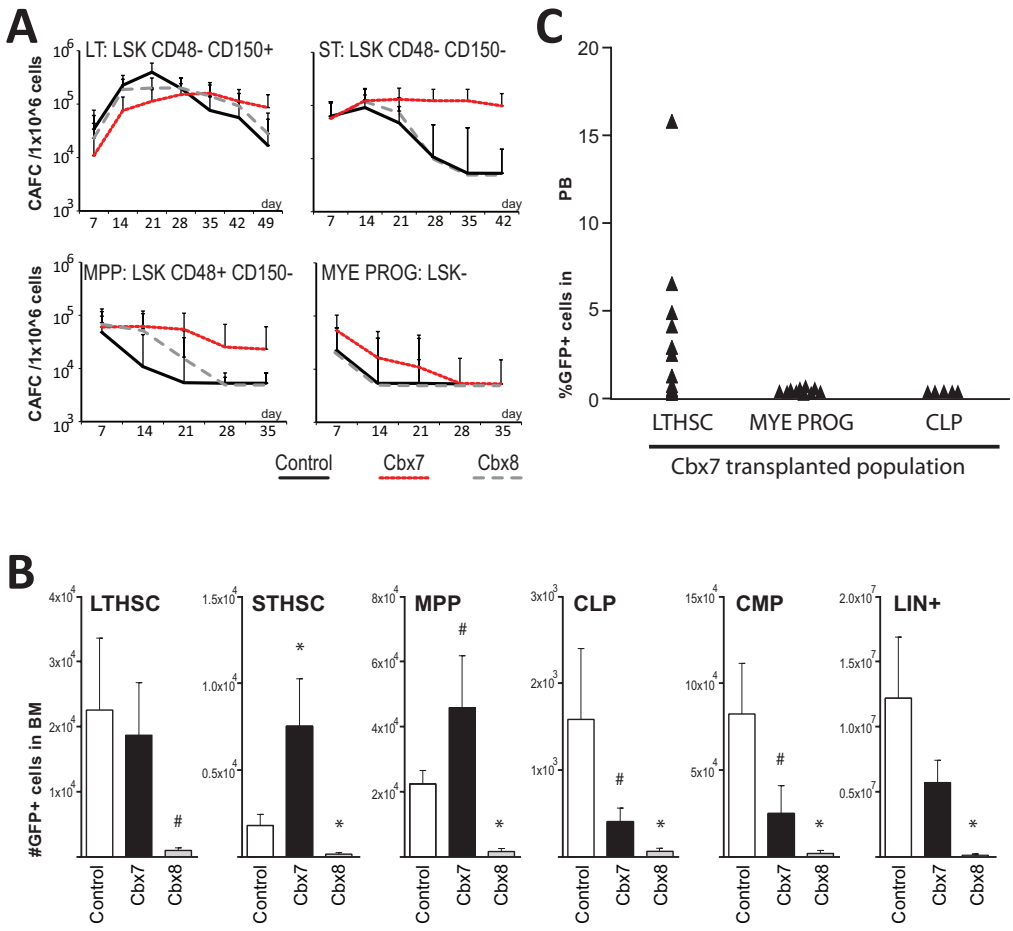


Figure 4. Cbx7 induces self-renewal of LT-HSCs, ST-HSCs and MPPs, but not of lineage restricted progenitors. A CAFC frequencies of purified LTHSCs, STHSCs, MPPs or myeloid progenitors, transduced with indicated vectors. Cells were plated in limiting dilution (1,3 and 10 cells/well), after which the average number of CAFC (± 95 per cent confidence interval) is calculated as described in [59]. **B** Phenotypical analysis of the bone marrow compartment of mice that were transplanted 12 months earlier with LT-HSCs that overexpress either Control (n=5), Cbx7 (n=5) or Cbx8 (n=3). Fig represents average (±s.e.m.) number of absolute GFP+ cells in the bone marrow of the indicated phenotype. n represents individual mice. t-test *p<0.05, #p<0.10. See Table S7 for raw data. **C** Chimerism levels as indicated by the percentage of GFP+ cells in the peripheral blood 24-weeks post transplantation. Mice were transplanted with either 750 Cbx7-overexpressing (GFP^+) LT-HSCs (LSK, $CD48^-$, $CD150^+$), myeloid progenitors (LSK^-,) or CLPs (Lin- Sca^{mid} cKitmid $CD127^+$). Symbols represent chimerism levels of individual mice.

ChIP-sequencing (ChIP-seq) of Flag-precipitated DNA was used to identify genome-wide Cbx7- and Cbx8-binding sites in primary HSPCs. Analysis of Cbx8 ChIP-seq relative to empty vector (control) revealed a total of 7649 peaks, of which 7091 peak positions could be annotated, mapping to 5590 genes (Supplementary Information). Although overall

sequencing depth was comparable (31.9x10^6 reads for Cbx7 versus 29x10^6 reads for Cbx8), substantially fewer peaks were identified for Cbx7; 3621 peaks were called, of which 3225 could be annotated, mapping to 2768 genes (Supplementary Information). Cbx7- and Cbx8-enriched regions are predominantly found at the transcription start site (Fig 7c,d).

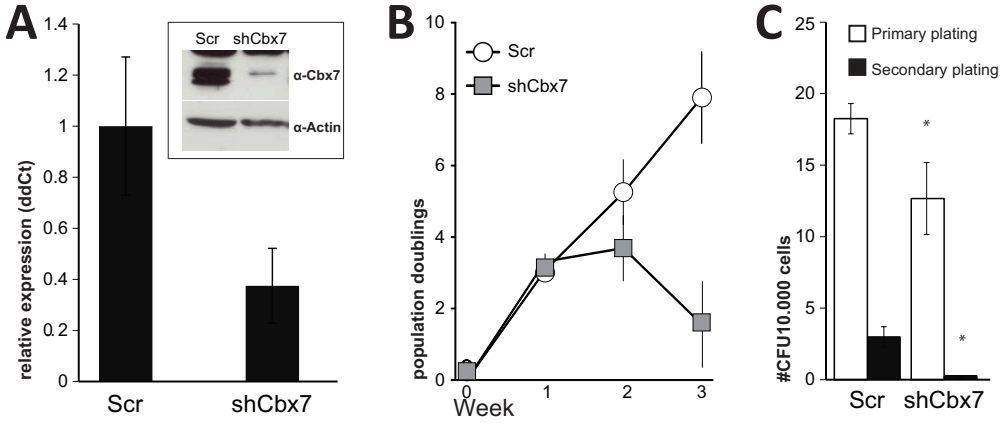


Figure 5. Cbx7 is required for HSPC self-renewal. A RT-qPCR analyses of Cbx7 mRNA expression in HSPCs transduced with Scrambled (Scr) and Cbx7 short hairpin (shCbx7) lentivirus. Expression was normalized to Hprt (*n*=3 independent experiments, mean±s.d.). Insert: Western blot analysis of HSPCs transduced with control and shCbx7 lentivirus. Actin was used as loading control. **B** Number of population doublings for HSPCs in which Cbx7 was downregulated (shCbx7) or HSPCs transduced with Scr lentiviral vector (n=3 independent experiments, mean±s.d., t-test p=0.08) **C** Colony-forming ability of Control (Scr) and Cbx7-downregulating HSPCs (CFU=colony forming unit, n=3 independent experiments, mean±s.d., t-test *p<0.05). See Table S7 for raw data.

Most identified targets were common for Cbx7 and Cbx8 (Fig 7e). Cross-comparison of Cbx8 with Cbx7 (using Cbx7 ChIP-seq signal as the background for Cbx8 peak calling), showed that of the 5590 Cbx8 target genes, only 291 genes were not bound by Cbx7, and thus are unique for Cbx8 (Fig 7e). Only 29 unique Cbx7 target genes were identified (Fig 7e).

We assessed the quantitative differences of Cbx binding by counting the number of tags per peak on shared Cbx7 and Cbx8 loci (Fig 7e) (Supplementary Information). As we used the same antibody for ChIP-seq and the total read counts were comparable between Cbx7 and Cbx8, we reason that the number of tags per peak reflects the strength of occupancy on a particular genomic location, although we cannot exclude some extent of technical noise. For most overlapping loci, the number of tags per peak was consistently twice as high for Cbx8 as for Cbx7, suggesting that Cbx8 has a stronger overall affinity towards chromatin binding. This notion is further supported by the fact that Cbx8 has more targets than Cbx7 (5590 vs. 2768 respectively). However, on some genomic positions (Fig 7e), corresponding to 186 genes, the number of tags per peak was significantly higher for Cbx7.

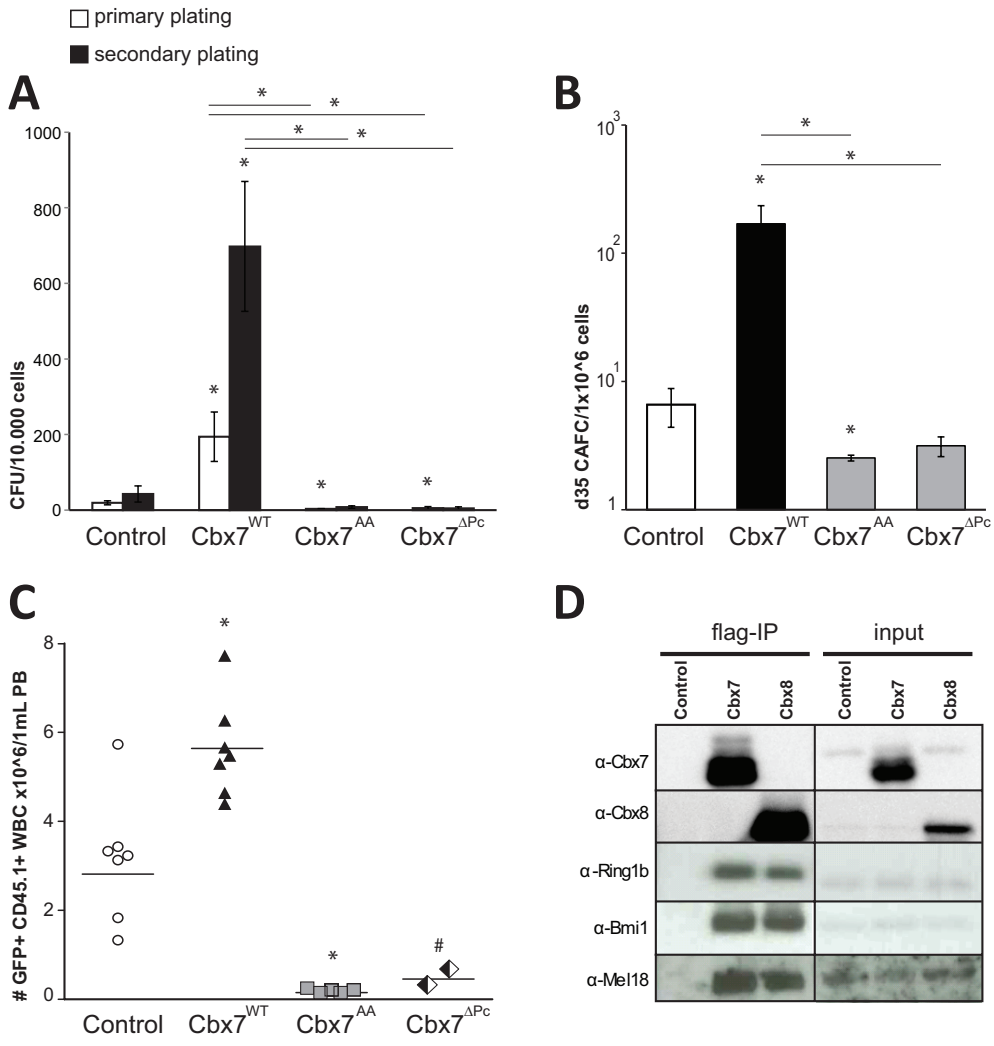


Figure 6. Cbx7-induced self-renewal requires PRC1 integration and H3K27me3 binding. A Primary and secondary colony forming ability of (post 5-FU) HSPCs transduced with control, Cbx7WT, Cbx7 chromodomain-mutant (Cbx7AA), and Cbx7 Pc-box deletion (Cbx7ΔPc) vectors (n=4 independent experiments, mean±s.e.m., t-test *p<0.05). See Table S7 for raw data. **B** Day-35 CAFC frequencies of control, Cbx7WT, Cbx7AA and Cbx7ΔPc bone marrow cells. n=4 independent experiments, mean±s.e.m., t-test *p<0.05. See Table S7 for raw data. **C** Post-transplantation chimerism levels as measured by the absolute number of GFP+ CD45.1+ donor cells in peripheral blood at week 12. Symbols represent chimerism levels of individual mice (Control n=7, Cbx7WT n=7, Cbx7AA n=5, Cbx7ΔPc n=2), line represents average chimerism levels. t-test *p<0.01, #p<0.06). **D** Endogenous Polycomb proteins bind ectopically expressed flag-Cbx7 and flag-Cbx8. Five percent of input was used as loading control. Uncropped images of blots are shown in Supplementary Figure S9b.

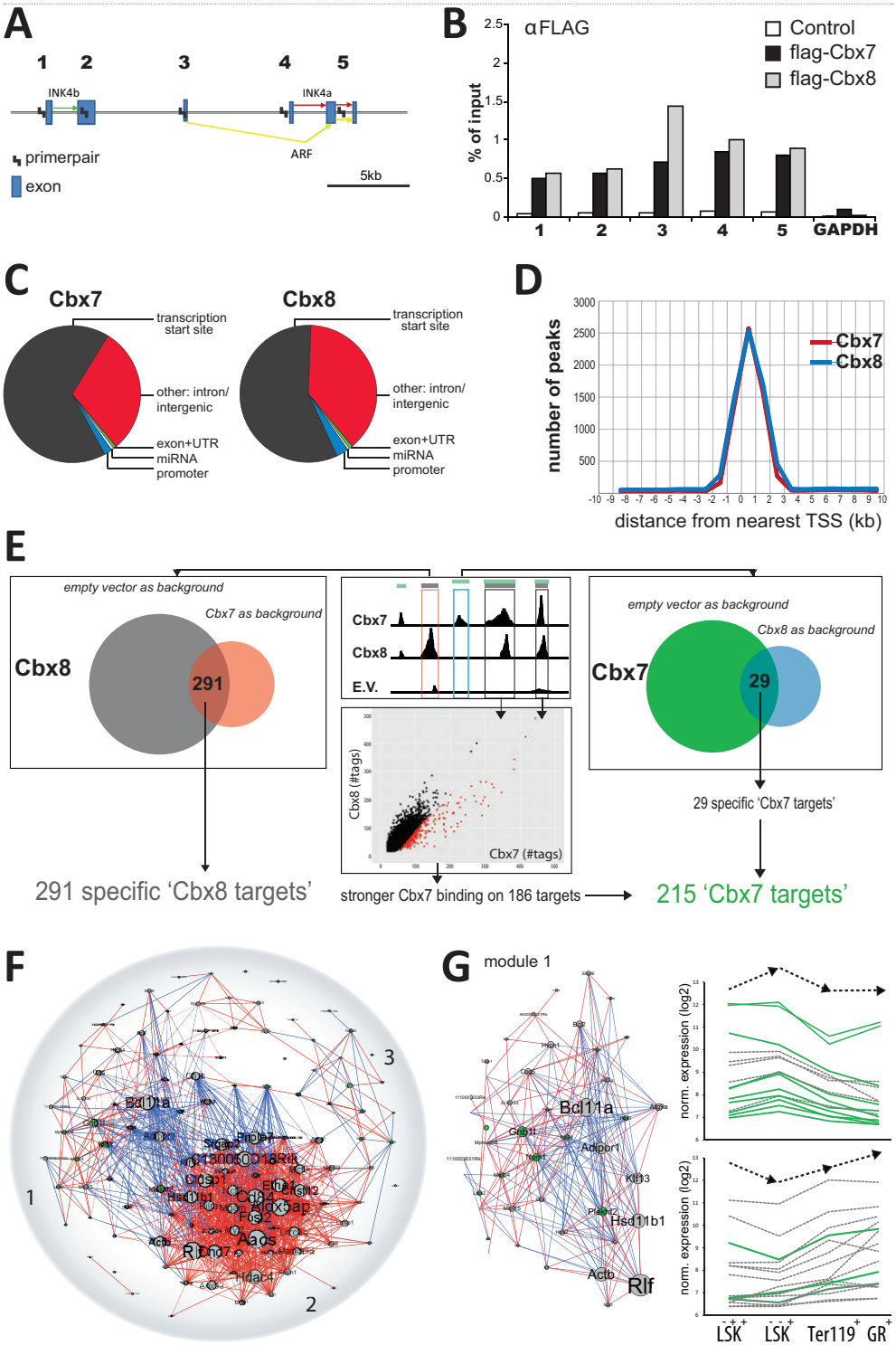
Collectively, Cbx7 and Cbx8 share most of their targets. Only 291 unique Cbx8 targets and 29 unique Cbx7 targets were identified. Together with the 186 targets that showed quantitatively stronger binding by Cbx7, these genes are likely to account for the opposite phenotypes observed on HSCs (Fig 7e, Table S2, Fig S9a,b). Differential Cbx7 and Cbx8 targets contain oncogenes, differentiation-specific genes, cell-cycle regulators and transcription factors (Table S3).

Cbx7 and Cbx8 targets are co-regulated during HSC differentiation

PcG-induced cell specification in the hematopoietic system is more likely orchestrated by changes in gene networks composed of signaling molecules, chromatin modifying complexes and transcription factors, rather than by changes in the expression of a few individual genes. Therefore, we investigated whether any of the Cbx7 and Cbx8 targets exhibit correlated expression changes along HSC differentiation.

Expression data for the 215 Cbx7 and 291 Cbx8 targets were extracted from published transcriptome data in which four distinct hematopoietic cell stages (HSCs, progenitors, erythroid precursors and granulocytes) in two genetically distinct strains of mice were profiled (23 different micro-arrays, n=5/6 for each cell population) [30]. From these data gene networks were constructed [31] consisting of 33 Cbx7 and 85 Cbx8 targets that collectively showed correlated expression along hematopoietic differentiation (Fig 7f, Table S4).

Three highly connected subnetworks (modules) were identified. (Fig 7f). In module 1, most genes that are coordinately repressed in HSC and become up-regulated in progenitors are Cbx7 targets (Fig 7g, Table S4). This is in agreement with the transcriptional repressive function of highly expressed Cbx7-PRC1 complexes in HSCs. Among them, *NPM1* is a regulator of the *ARF/p53* pathway, and loss-of-function mutations, deletions and translocations have been associated with acute myeloid leukemia [32-33]. Within the same module, genes that show reciprocal expression changes during differentiation are mostly Cbx8 targets. These targets become repressed upon the transition from HSCs into progenitors. *Bcl11a* and *Rlf* show many connections with other genes in this module, and are therefore putative major regulators. *Bcl11a* expression is found to be down-regulated during differentiation, and its overexpression has been associated with leukaemogenesis [34-35].


A
1 2 3 4 5
INK4b
INK4a
ARF
primerpair
exon
5kb
B
αFLAG
Control
flag-Cbx7
flag-Cbx8
% of input
2.5
2
1.5
1
0.5
0
1 2 3 4 5 GAPDH
C
Cbx7
transcription start site
other: intron/ intergenic
exon+UTR
miRNA
promoter
Cbx8
transcription start site
other: intron/ intergenic
exon+UTR
miRNA
promoter
D
number of peaks
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2500
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1000
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Cbx7
Cbx8
-10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
distance from nearest TSS (kb)
E
empty vector as background
Cbx7 as background
Cbx8
291
291 specific 'Cbx8 targets'
Cbx7
Cbx8
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Cbx8 (#tags)
Cbx7 (#tags)
stronger Cbx7 binding on 186 targets
empty vector as background
Cbx8 as background
Cbx7
29
29 specific 'Cbx7 targets'
215 'Cbx7 targets'
F
1
2
3
Bcl11a
Rlf
Aacs
G
module 1
Bcl11a
Rlf
Actb
Hsd11b1
Kit13
norm. expression (log2)
LSK
LSK
Ter119
GR

Figure 7. Cbx7 and Cbx8 targets are coordinately regulated during HSC differentiation and show reciprocal expression patterns. A Schematic representation of the mouse *Ink4b-Ink4a-Arf* locus and primer pairs used for chromatin immunoprecipitation (ChIP) analysis in (B). **B** Binding of Cbx7 and Cbx8 to the *Ink4b-Ink4a-Arf* locus assessed by flag-ChIP. Bars represent mean of 2 independent experiments. See Table S7 for raw data. **C** Diagram illustrating the overall distribution of Cbx7 and Cbx8 binding sites on TSS (±1 kb), promoter (-1 to -5 kb), miRNA, exon+UTR, intron and intergenic regions. **D** Localization of Cbx7 and Cbx8 ChiP-seq peaks relative to nearest TSS. **E** Schematic representation illustrating data analysis of flag ChiP-sequencing. **F** Graphic representation of the gene network of Cbx7 and Cbx8 targets that show correlated gene expression changes during HSC differentiation. Expression of the 215 Cbx7 and 291 Cbx8 target genes was extracted from transcriptome data of four distinct hematopoietic cell populations (HSCs ($L^-S^+K^+$), progenitors ($L^-S^-K^+$), erythroid cells ($Ter119^+$) and granulocytes ($Gr1^+$). Green nodes represent Cbx7 targets, grey nodes represent Cbx8 targets. Node size corresponds to the number of significant correlations of gene expression of a particular gene with other genes. Lines between nodes represent positive (red) or negative (blue) correlations of gene expression during hematopoietic differentiation. **G** Module 1 within the network represents one of the strongest connections within the total network. Right panels: Genes (represented by nodes) are clustered on basis of similarities in gene expression profiles and plotted in two graphs that show an inverse expression pattern along hematopoietic differentiation ($L^-S^+K^+$ HSCs, $L^-S^-K^+$ progenitors, erythroid cells ($Ter119^+$), granulocytes (Gr^+)). Green lines represent expression changes of Cbx7 targets, grey dotted lines of Cbx8 targets. Raw expression data can be found in Table S4.

Module 2 represents a sub-network (Fig S8c) of genes specific for myeloid differentiation that almost exclusively consists of Cbx8 targets. These genes show repression upon the transition from HSCs into progenitors and become highly up-regulated during myeloid differentiation (Fig S8c, Table S4). This myeloid network is negatively regulated by a single Cbx8 target; *Gfi1b*. *Gfi1b* is a well-known transcription factor and plays a critical role in the control of erythropoiesis and megakaryopoiesis [36-38]. Strikingly, two Cbx7 targets, *Sfpq* and *Ube3c*, also negatively regulate the Cbx8 myeloid specific gene network and show reciprocal gene expression patterns (Fig S8c).

Finally, module 3 contains genes differentially expressed in erythroid cells. Genes that become down-regulated during differentiation from stem to progenitor to erythroid cells represent predominantly Cbx8 targets, whereas genes with a reciprocal expression pattern are enriched for Cbx7 targets (Fig S8d, Table S4).

Our approach shows that Cbx7 and Cbx8 targets are negatively co-regulated during HSC specification. Generally, genes bound only by Cbx8 are higher expressed in HSCs and become repressed during differentiation into progenitors, indicating that Cbx8 selectively targets HSC-specific genes. In turn, Cbx7 targets show the opposite expression pattern. Therefore, Cbx7 might induce HSC self-renewal by repressing progenitor-specific genes.

DISCUSSION

During evolution, the number of genes encoding Polycomb proteins have expanded, which has resulted in structural and functional diversification of PRCs. Here, we studied the role of different PRC1-associated Cbx family members in HSC regulation. Cbx family members show distinct hematopoietic cell-stage-specific expression patterns, which allows for the formation of variant PRC1 complexes during hematopoietic differentiation. As Cbx7 is highest expressed in the most immature hematopoietic populations, the PRC1 complex in HSCs preferentially contains Cbx7. PRC1 complexes containing Cbx7 induce self-renewal of HSCs by repressing the expression of progenitor-specific genes. Other Cbx proteins can compete with Cbx7, resulting in Cbx2-, Cbx4-, or Cbx8-containing PRC1 complexes that target HSC-specific genes and thereby induce entrance into the differentiation pathway. We suggest that stoichiometric fluctuations between PRC1 complexes containing different Cbx family members play a key role in balancing self-renewal and differentiation of HSCs (Fig S1).

Diversification of Polycomb genes might also account for tissue-specific stem cell functioning. Whereas we show that Cbx7 is the most important Cbx family member in HSCs, in epidermal stem cells Cbx4 plays this essential role [39]. It remains to be determined which of the Cbx family members are required for other types of adult stem cells. Recently, Cbx7 has been identified as an embryonic stem cell (ESC) pluripotency factor [8,10]. So far, genes that have been associated with ESC pluripotency, including Oct4 and Nanog, have been very distinct from genes required for self-renewal in adult stem cells. To our knowledge Cbx7 is the first gene important for self-renewal in both ESC and adult stem cells, which indicates that the molecular mechanism by which it exerts its activity might be conserved during ontogenesis.

In search for such a mechanism, we compared targets that we identified in HSCs with Cbx7 targets previously identified in ESCs [8]. This ESC-HSC cross-comparison revealed 600 common targets which represent approximately 25% of all Cbx7 targets in ESCs (2349) [8] and HSCs (2768) (Table S5). Interestingly, we identified 133 transcription factors, many of which are members of the same family (e.g. 12 *Fox* genes, 14 *Hox* genes and 3 *Sox* genes) (Table 1, Table S6). In addition, Cbx7 ECS-HSC targets include *Fgf* and *Eph* receptors and genes involved in Wnt signaling. We hypothesize that Cbx7-regulated control of these genes is a core element of self-renewal.

Our data support the notion that the balance between self-renewal and differentiation, which constitutes a key hallmark of HSCs, is regulated by the molecular composition of the PRC1 complex. Therefore, genetic or pharmacologic perturbation of the composition of PRC1 may provide a strategy to induce or repress self-renewal in normal or aberrant hematopoiesis.

Table 1: GO-analysis of genes targeted by Cbx7 in both ESCs and HSCs

GO ID	Molecular function	Total genes in GO	Matched genes (of 600 total)	corrected p-value Benjamini FDR	Gene families *
GO:0003700	sequence-specific DNA binding transcription factor activity	706	133 (18,84%)	4.10E-78	Dlx(3), Fox(12), Hox(14), Lhx(6), Nr(5), Pitx(3), Six(3), Sox(3), Tbx(3)
GO:0008270	zinc ion binding	1334	59 (4,42%)	2.24E-04	Lhx(6), Prdm(4), Sp(3), Zic(3)
GO:0003682	chromatin binding	249	19 (7,63%)	5.06E-04	Dlx(2), Gata(2)
GO:0004714	transmembrane receptor protein tyrosine kinase activity	49	9 (18,37%)	1.27E-04	Fgf(2), Eph(2)
GO:0005102	receptor binding	236	17 (7,2%)	2.60E-03	Wnt(6)
GO:0003707	steroid hormone receptor activity	47	7 (14,89%)	4.75E-03	Nr(5)
GO:0017147	Wnt-protein binding	26	5 (19,23%)	8.86E-03	Fzd(3)

* numbers in parentheses represent the number of identified gene family members

ACCESSION NUMBERS

Primary accessions: ChIP-seq data files are available at the Gene Expression Omnibus (GEO) under GSE36658. Referenced accession: GSE35292.

Supplementary Information is linked to the online version of the paper at *www.nature.com/nature*

Figure S9: uncropped western blot images
Supplementary Table 1: Cbx7-induced leukemic mice.
Supplementary Table 2: Differential Cbx7 and Cbx8 targets
Supplementary Table 3: Gene-ontology analysis of differential Cbx7 and Cbx8 targets
Supplementary Table 4: Cbx7 and Cbx8 targets show reciprocal expression patterns during HSC differentiation
Supplementary Table 5: Cbx7 targets in HSCs overlap with Cbx7 targets in ESCs
Supplementary Table 6: Gene-ontology analysis of common Cbx7 targets in ESCs and HSCs.
Supplementary Table 7: raw data
Supplementary Data 1: ChIP-seq analysis of Cbx7 and Cbx8 binding sites.

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AUTHOR CONTRIBUTIONS

K.K., L.V.B., K.H., and G.d.H. initiated research and developed the concept of the paper. K.K., L.V.B., and G.d.H. designed research; K.K. and V.R. performed experiments with contributions from M.B., E.W., S.O., M.R., S.B., and X.W; E.Z. performed bioinformatics analyses with contributions from L.V.B. and K.K.; K.K. and L.V.B. analyzed and interpreted data; and K.K. wrote the manuscript with contributions from L.V.B., K.H., and G.d.H.

SUPPLEMENTARY FIGURES

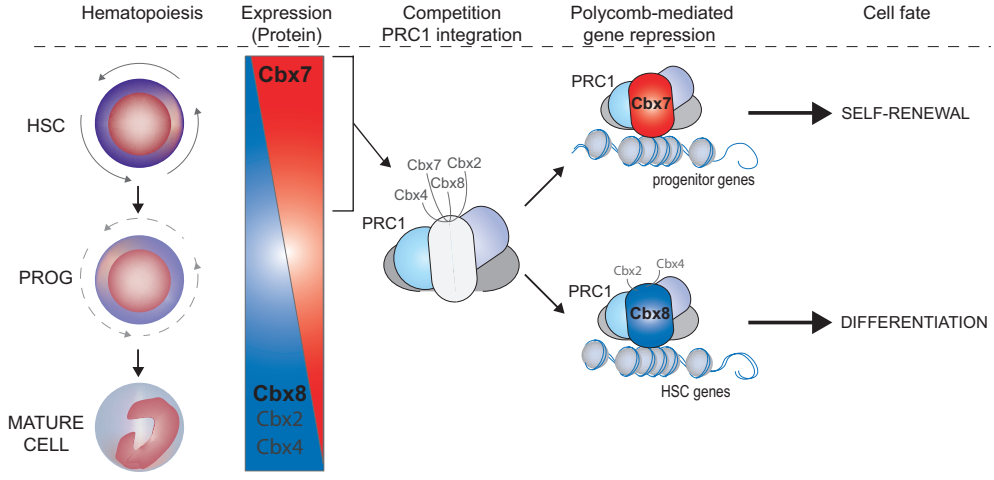

Figure S1. PRC1-Cbx control of HSC fate decisions. Model summarizing the findings in this paper. Polycomb Cbx orthologs compete for PRC1 integration and balance HSC self-renewal and differentiation. Cbx7-containing PRC1 complexes induce self-renewal of HSCs by repressing the expression of progenitor-specific genes. Other Cbx proteins can outcompete Cbx7 from PRC1, resulting in Cbx2-, Cbx4-, or Cbx8-containing PRC1 complexes that target HSC-specific genes and thereby induce entrance into the differentiation pathway.

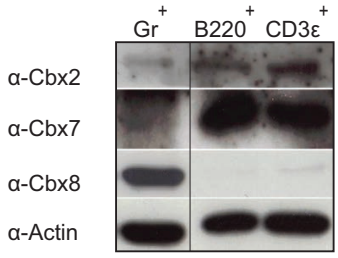

Figure S2. Protein abundance of Cbx orthologs in differentiated hematopoietic cells. Western blot analysis of protein abundance of Cbx2, Cbx7 and Cbx8 in granulocytes (Gr1+), B cells (B220+) and T-cells (CD3e+). Actin was used as loading control. Also see Figure S9.

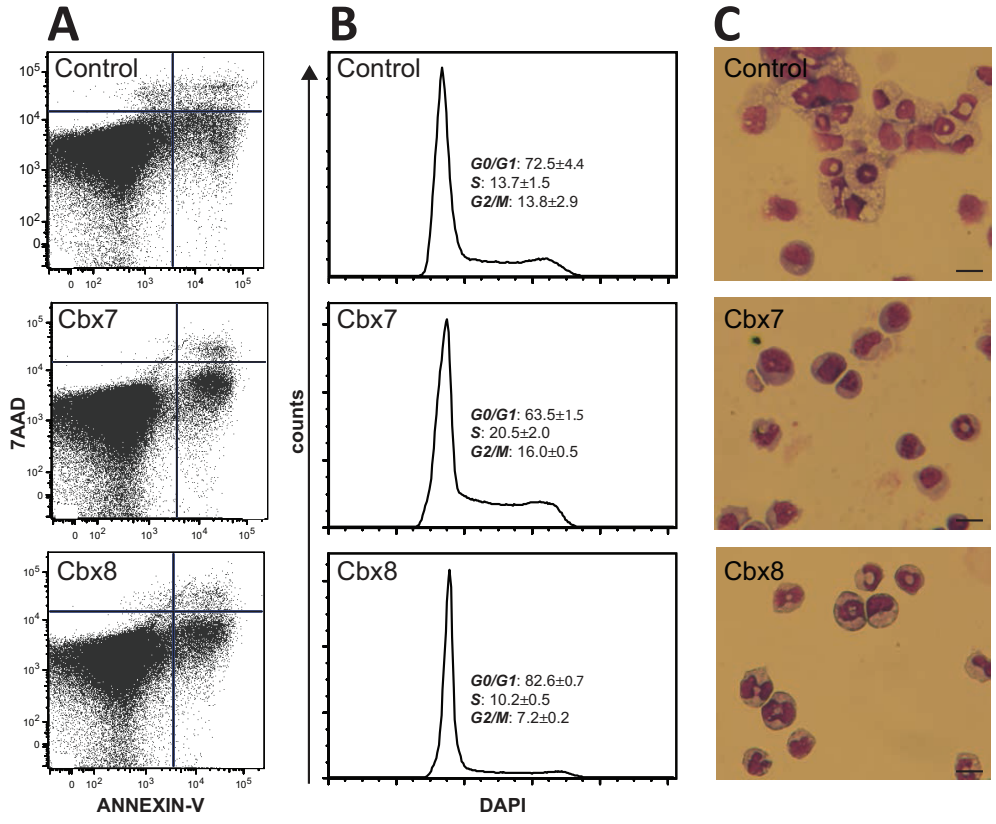

Figure S3. Effect of Cbx7 and Cbx8 overexpression on the cell-cycle. Post 5FU treated bone marrow cells were transduced with Control or Cbx-overexpressing retroviral vectors. GFP+ HSPCs were plated in cytokine-supplemented media and at day 10 cells were used for apoptosis assays, cell-cycle analysis and cytospins. **A** Apoptotic frequency of control, Cbx7 and Cbx8 bone marrow cells using FACS analysis of annexin-V and 7AAD positive cells. One representative FACS plots is shown from 3 independent experiments. **B** Cell cycle analysis by measurement of DNA content in control, Cbx7 and Cbx8 HSPCs (n=2 independent experiments, mean±s.d.). **C** May-Grünwald Giemsa staining of control, Cbx7 and Cbx8 cells. One representative image is shown. Scale bar 10μM. Also see Table S7 for raw data.

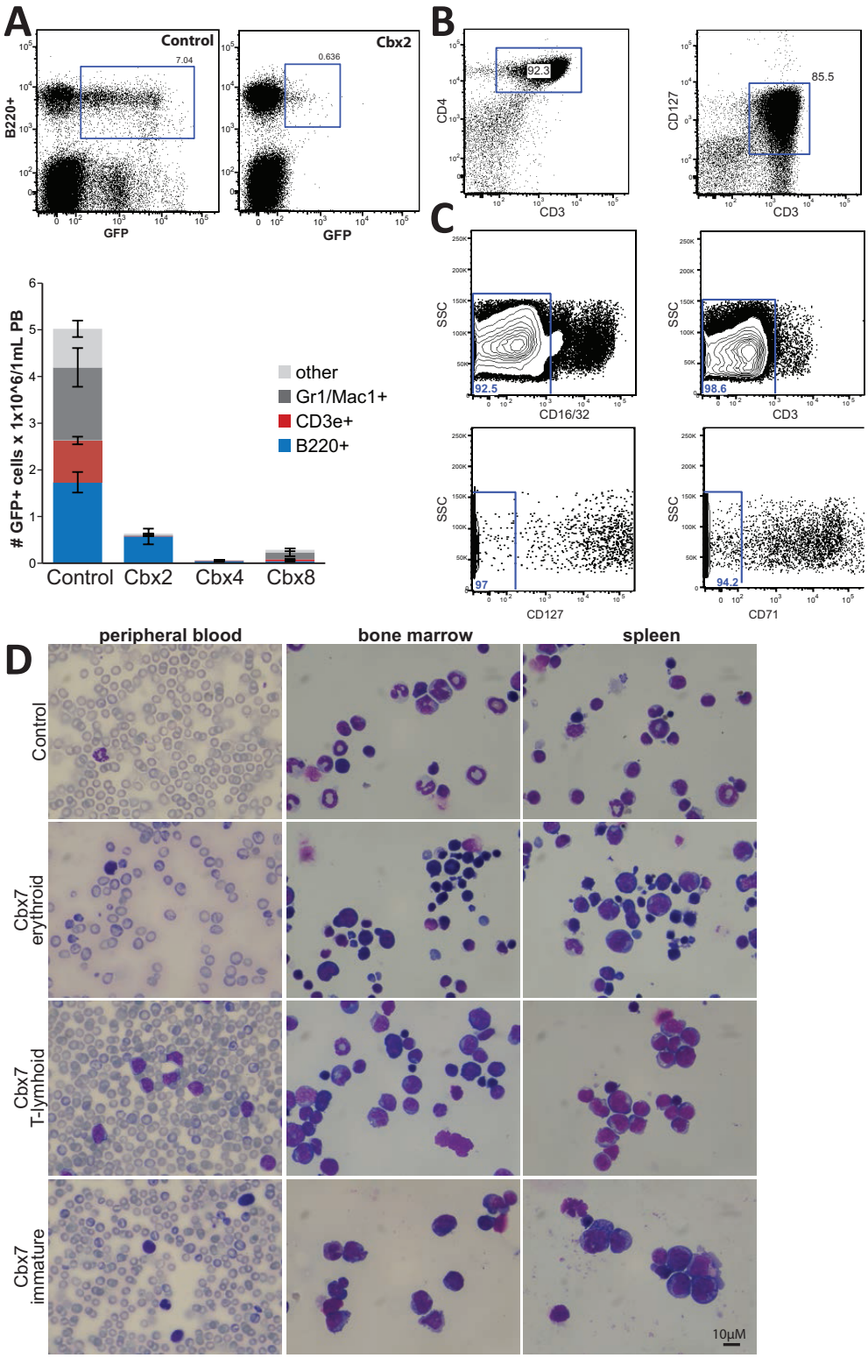
A
Control
Cbx2
7.04
0.636
B220+
GFP
GFP+ cells x 1x10^6/1mL PB
other
Gr1/Mac1+
CD3e+
B220+
Control
Cbx2
Cbx4
Cbx8
B
92.3
85.5
CD4
CD127
CD3
C
92.5
98.6
97
94.2
SSC
CD16/32
CD3
CD127
CD71
D
peripheral blood
bone marrow
spleen
Control
Cbx7 erythroid
Cbx7 T-lymhoid
Cbx7 immature
10μM

Figure S4. Cbx7-induced leukemia subtypes. A Analysis of lineage contribution of transplanted HSPCs transduced with Control, Cbx2, Cbx4 and Cbx8 retrovirus, 16 weeks post-transplantation. n=7-9 individual mice, mean±s.e.m. **B** FACS plot example of spleen cells from a mouse (mouse9 suppl table 1) with (CD3+, CD4+, CD127+) T-cell leukemia. Cells within CD45.1+, GFP+ fraction are shown. **C** Staining of spleen cells of mouse 14 (suppl table 1) with an immature leukemia type with CD3, CD127, CD71 and CD16/32. Cells within CD45.1+, GFP+ fraction are shown. **D** May-Grunwald-Giemsa staining of cells in peripheral blood, bone marrow, and spleen of control mice and Cbx7 mice with indicated leukemia subtypes. One representative image is shown for every condition. Scale bar 10μM.

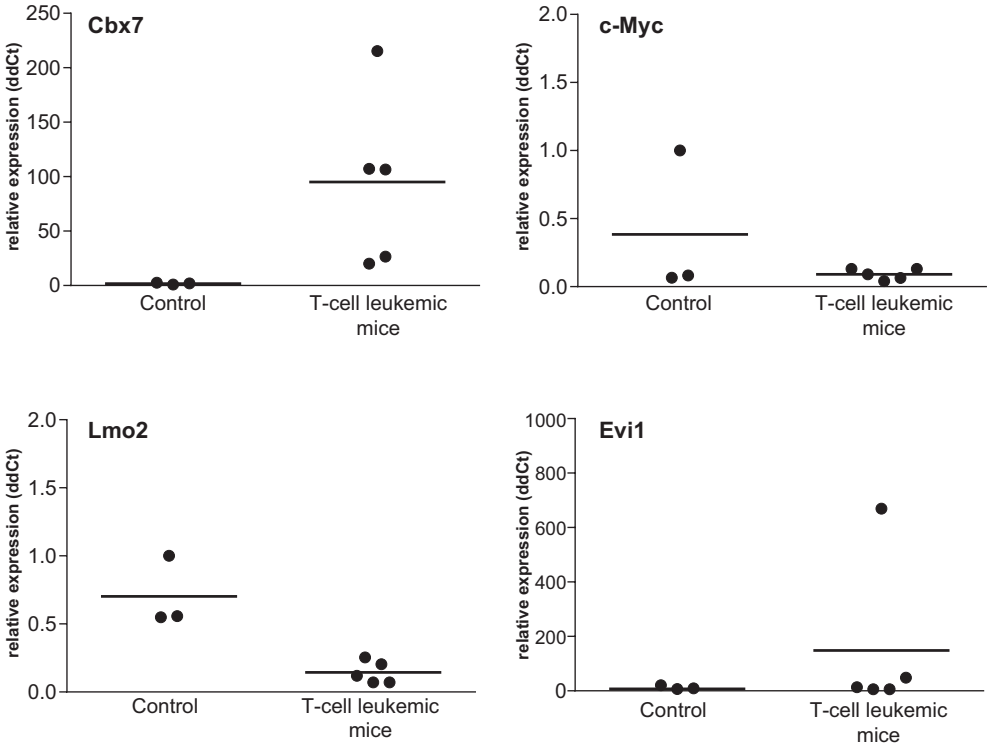

Figure S5. Oncogene expression in Cbx7-induced lymphoid leukemias. Relative gene expression of Cbx7, c-Myc, Evi-1, Lmo2 in spleen cells from individual Control (n=3) and T-cell leukemic mice (n=5). Gapdh was used for normalization.

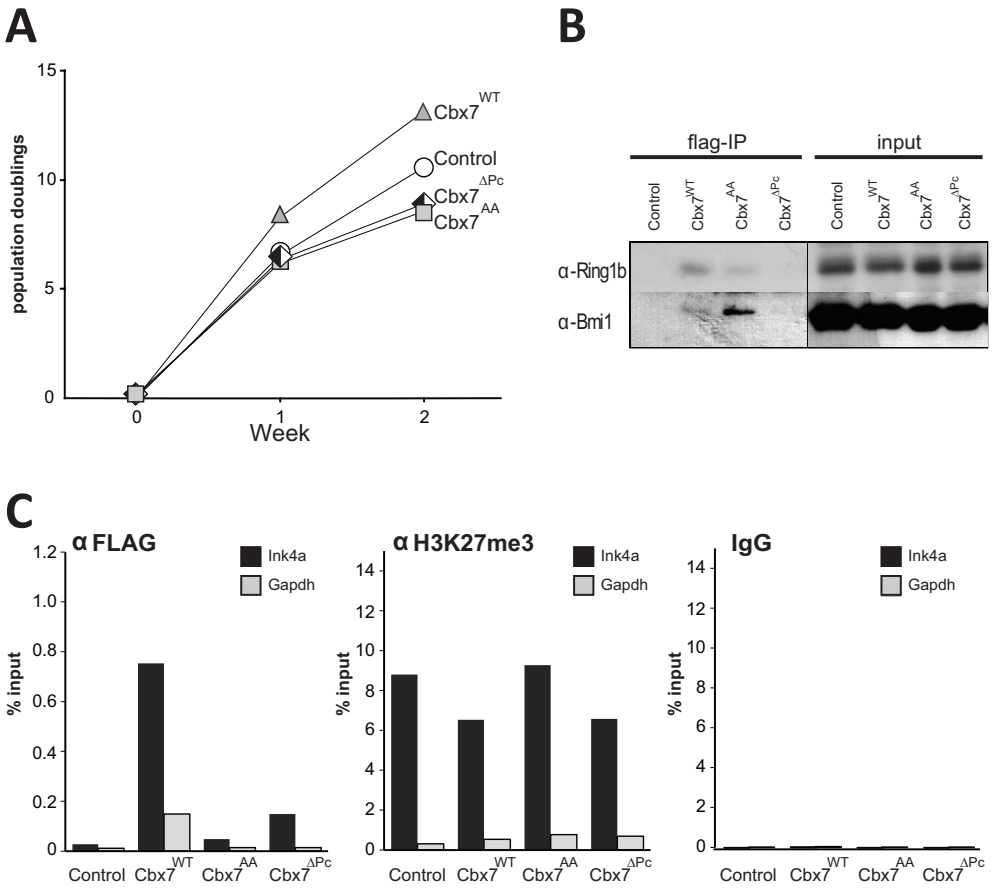

Figure S6. Cbx7-induced HSC self-renewal is Polycomb dependent. A Population doublings of HSPCs overexpressing Cbx7WT, flag-Cbx7AA, and flag-Cbx7ΔPc . Bars represent mean from 2 independent experiments. **B** Flag-immunoprecipitation of Ring1b and Bmi1 with flag-Cbx7WT, flag-Cbx7AA, and flag-Cbx7ΔPc in 32D cells. One representative experiment is shown from two independent experiments. Also see Figure S9. **C** Binding of Cbx7WT, flag-Cbx7AA, and flag-Cbx7ΔPc to the transcription start site of Ink4a. HSPCs were transduced with indicated vectors. Chromatin-immunoprecipitation was then performed using Flag-M2 agarose beads and H3K27me3 antibodies coupled to sepharose beads. IgG was used as negative control. Pulled down DNA was purified and binding to Ink4a was assessed using qPCR. Bars represent mean from 2 independent experiments. Also see Table S7 for raw data.

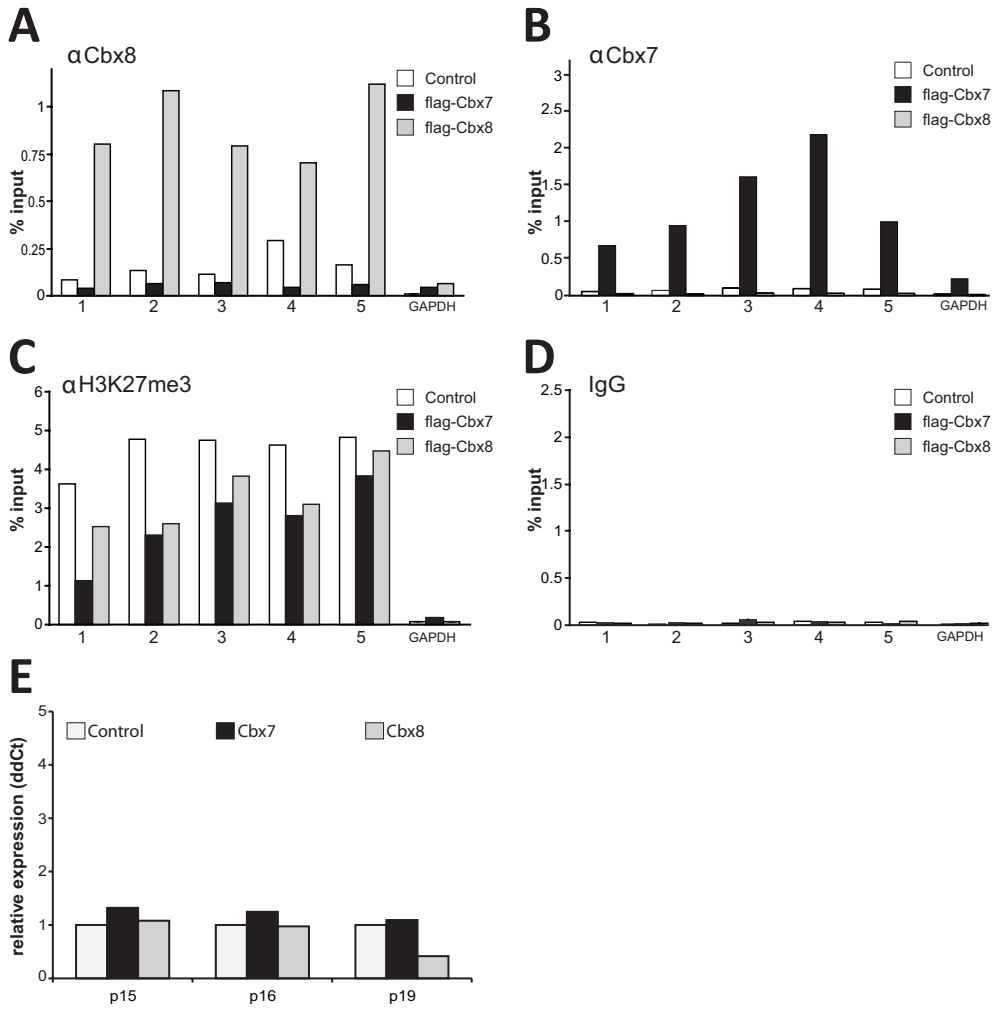

Figure S7. Cbx7 and Cbx8 bind the senescence locus. A-D Post-5FU treated bone marrow cells transduced with Control or Cbx-overexpressing retroviral vectors were sorted based on GFP expression and cultured for 7 days in cytokine-supplemented media. Next, ChIP was performed in control, Cbx7 and Cbx8 bone marrow cells using indicated antibodies. Numbers correspond to the Ink4b-Ink4a-Arf primer pairs used for qPCR, as shown in Figure 4a. Bars represent mean from 2 independent experiments. **E** Relative expression of p15, p16 and p19 after Cbx7 and Cbx8 overexpression in bone marrow compared to control, as determined by rt-PCR. Expression was calculated according to ddCT of Gapdh. Bars represent mean from 2 independent experiments. Also see Table S7 for raw data.

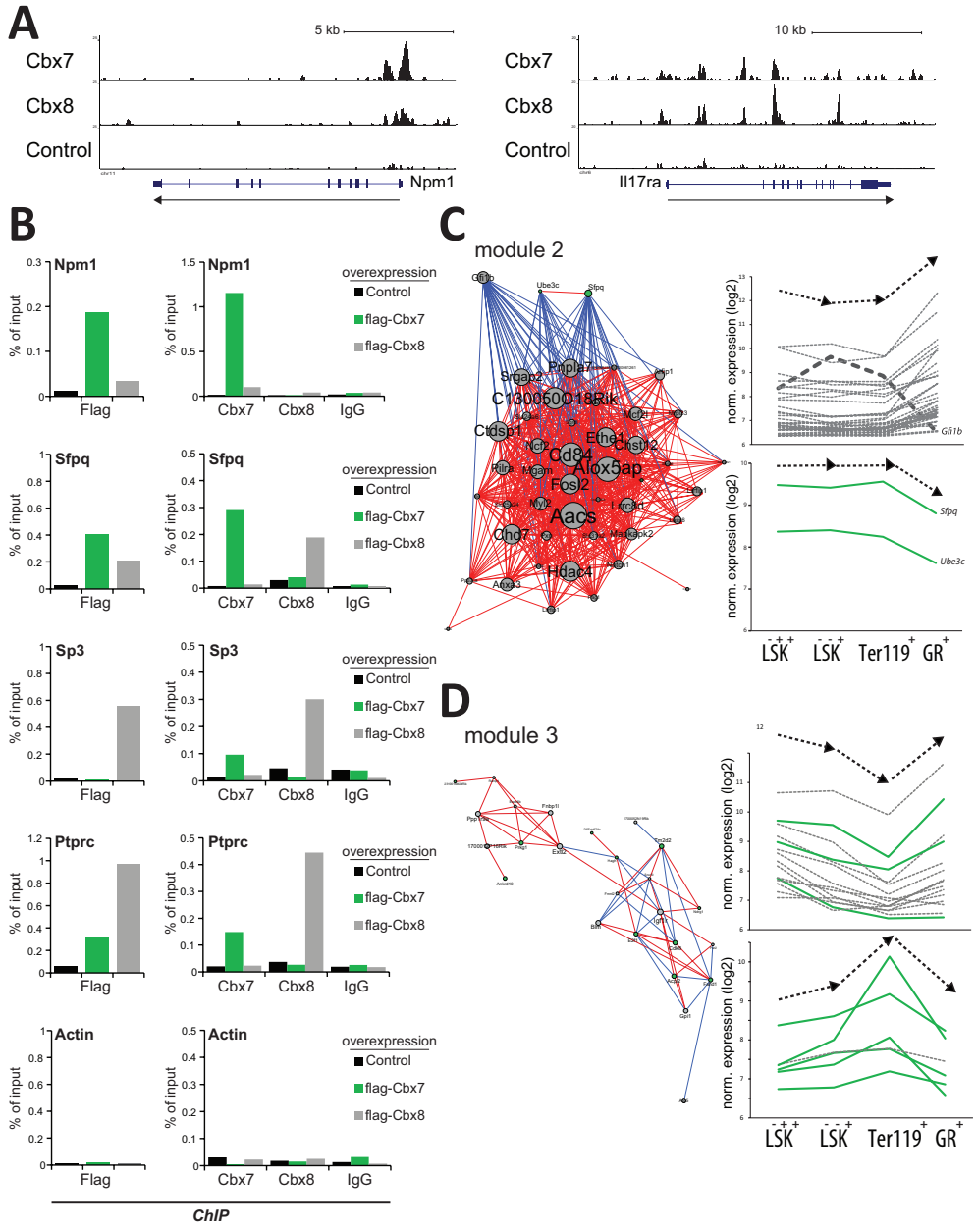

Figure S8. Cbx7 and Cbx8 targets and gene network. A Representative examples of ChIP-seq results in Control, Cbx7-overexpressed and Cbx8-overexpressed bone marrow cells. The y-axis of the binding profiles indicates tag counts. **B** Validation of ChIP-seq targets by flag-ChIP, Cbx7-ChIP and Cbx8-ChIP followed by qpcr. Flag-ChIP and Cbx-ChIPs represent independent experiments. Npm1 and Sfpq are Cbx7 targets (either not bound by Cbx8, or stronger bound by Cbx7 than by Cbx8), Sp3 and Ptprc are specific Cbx8 targets. Actin was used as a negative control locus. Also see Supplementary Table 7 for raw data. **C** Module 2 represents a myeloidspecific sub-network within the network. D) Module 3 represents an erythroid specific sub-network. Right panels of **C** and **D**: Genes (represented by nodes) are clustered on basis of similarities in gene expression profiles and plotted in two graphs that show an inverse expression pattern along hematopoietic differentiation (L-S+K+ HSCs, L-S-K+ progenitors, erythroid cells (Ter119+), granulocytes (Gr+)). Green lines represent expression changes of Cbx7 targets, grey dotted lines of Cbx8 targets. Raw expression data can be found in Table S4.

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Chapter 5

The dynamic behavior and cellular evolution of multilineage leukemias induced by the Polycomb group protein Cbx7

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ABSTRACT

Our current understanding of the dynamics of tumor development and heterogeneity is still very limited, although this is highly relevant for existing and future therapeutic interventions. Although currently under debate, tumors were long thought to evolve in a linear fashion, as a single cell acquired progressive mutations that advanced its proliferative rate. In this study, using a barcoding tool combined with overexpression of a novel (proto-) oncogene, Cbx7, we show that the origin and nature of leukemias is more complex than previously anticipated. Different clones with distinct leukemic properties can coexist in a single mouse, resulting in oligoclonal leukemias. We document the existence of dormant clones with leukemic potential. These dormant LSCs can progress to full-blown leukemia at 'relapse', after serial transplantations. In addition, we show that some LSC clones retain multilineage differentiation capacities, where one LSC clone can induce multiple phenotypically distinct leukemias at 'relapse'. Thus, phenotypically distinct leukemias can have similar clonal origins. Clinically, the existence of multiple leukemic clones, with distinct biological behavior, suggests that combination therapy, rather than single-agents will be required to eradicate both dominant and dormant clones that otherwise may emerge at relapse.

INTRODUCTION

Leukemias are clonal proliferative diseases that arise from HSCs or progenitors that failed to obey normal regulatory signals which should restrict their self-renewal. Sequential 'linear' acquisition of mutations in tumor suppressor genes or oncogenes has long been thought to drive leukemogenesis, as postulated in the original clonal evolution hypothesis [1-3]. However, the use of advanced genomic techniques to investigate clonal diversity and evolution, now shows that tumors might be organized in a non-linear, branching hierarchy [4-8].

Only a subfraction of cells within the leukemic cell population, termed leukemic stem cells (LSCs), or leukemia-initiating cells (LICs), possesses the ability to initiate and sustain disease [9-10]. The first publications demonstrating heterogeneity within the LSC population utilized the detection of viral integration sites after transduction of acute myeloid leukemia (AML) cells, and showed that LSCs differ in their self-renewal capacities after transplantation in immunodeficient mice [11]. More recently, Anderson *et al*., employed fluorescence in situ hybridization (FISH) to monitor translocations and copy number alterations to investigate genetic heterogeneity within pediatric acute lymphoblastic leukemia (ALL) [6]. By assembling a putative ancestral evolutionary tree, encompassing the frequencies of genetic abnormalities found in a patient sample, they concluded that clones displayed variegated genetic abnormalities, which are likely to be inherited in a non-linear branching manner 6. Although the resolution of this FISH-based method to identify subclonal heterogeneity remained limited, it clearly provided a paradigmatic proof-of-principle. Deep sequencing genomic DNA or RNA (exome sequencing) of malignant cells from patients, provides the highest possible resolution for identification of mutations or other genetic abnormalities within a tumor. Such attempts have been made to assess clonal heterogeneity in leukemic patients [12-14]. Although potentially very powerful, these large datasets require the establishment of mathematical models and complex statistics to define subclones within populations of cells. Such mathematical models require the assumption of genetic stability of mutations and translocations, while genomes of cancers are generally instable. In addition, it remains difficult to distinguish which genetic abnormalities are causal to disease progression and which are irrelevant passenger mutations. Indeed, many mutations that may be used to identify subclones, have been suggested to be irrelevant for disease-progression [15]. Although these studies all highlight a potential complex genetic architecture of leukemia, unambiguous detection of leukemic clones remains difficult to achieve.

One of the most important questions in understanding leukemic progression remains the nature and number of different LSCs, and their subclones within an individual cancer. Next, it remains unknown to what extent different subclones can contribute to disease progression and potential relapse. It has been shown that some LSCs might be quiescent [11], while these formerly quiescent cells can become active at relapse. It is now generally believed that distinct leukemic clones exhibit

a distinct response to therapeutic interventions, reinforcing the relevance to assess the functional consequences of clonal heterogeneity within a leukemia. In order to approach this question, distinct subclones must be distinguished during disease development with (ultra-)high resolution. A recently described method, which would potentially accomplish exactly this, is the marking of cells by the introduction of a unique, heritable mark that can be detected in its offspring experimentally [16-21]. These methods rely on the viral integration of a random "barcode" sequence of fixed length in the genome of target cells. After transplantation of barcoded cells, the population and their offspring can be traced by quantifying the abundance of unique barcodes using deep sequencing.

Very recently, we have shown that retroviral overexpression of the Polycomb PRC1 member Cbx7 in bone marrow cells causes leukemia [22]. While typically a single oncogene causes one specific tumor type, the epigenetic modifier Cbx7 causes a wide spectrum of leukemias including T-ALL, erythroid and undifferentiated leukemias. By implementation of a barcoded Cbx7 overexpression vector, we have now generated a mouse model in which Cbx7 serves as the initial leukemic 'hit' and every pre-LSCs is uniquely labelled. We show how our approach allows for the identification of LSC-derived clones in the transplanted primary and secondary recipients. We describe clonal dynamics in mice that will succumb to leukemia. In addition, we document that minor clones with different leukemic potential can co-exist in a relatively clonal leukemia and that clonally-related leukemic cells can show lineage conversion. Both phenomena can result in variegated disease. Beyond a detailed insight into Cbx7-driven leukemic biology, our model will be highly relevant for the study of tumor dynamics and clonal evolution.

METHODS

Mice

C57Bl/6 (CD45.2) were purchased from Harlan. C57Bl/6.SJL (CD45.1) mice were bred, and all animals were housed at the Central Animal Facility of the University of Groningen. All animal experiments were approved by the Groningen University Animal Care Committee.

Construction of barcoded vector libraries

The SF91 vector was kindly provided by Prof C. Baum (Hannover Medical School, Hannover, Germany). The barcode linker was created by annealing two 5'-phosphorylated primers (Biolegio) with appropriate overhangs [19]: BsrGI-BamHI forward 5'-GTACAAGTAANNATCNNGATSSAAANNGGTNNAACNNTGTAAAACGACGGCCAGTGAG-3'; reverse,
5'-GATCCTCACTGGCCGTCGTTTTACANNGTTNNACCNNTTTSSATCNNGATNNTTACTT-3';).
BsrGI-Cla1 forward,
5' GTACAAGTAANNATCNNGATSSAAANNGGTNNAACNNTGTGGAACGACGGCCAGTGAAT-3',

reverse 5'- CGATTCACTGGCCGTCGTTCCACANNGTTNNACCNNTTTSSATCNNGATNNTTACTT-3'.
BamHI-BstBI forward
5' GATCCAGTAANNATCNNGATSSAAANNGGTNNAACNNTGTCAAACGACGGCCAGTGAAT-3',
reverse 5'-CGATTCACTGGCCGTCGTTTGACANNGTTNNACCNNTTTSSATCNNGATNNTTACTG-3'.
Primers were dissolved in 0.5× ligation buffer (Fermentas) at a concentration of 100µM. After heating the mixture for 5 minutes at 95°C, primers were allowed to anneal at gradually decreasing temperature. The annealed barcode linker was ligated into the BsrGI-BamHI site of the SF91-empty vector (Figure 1A), or the Cla1-BsrGI or in BamHI-BstBI site of the SF91-Cbx7 vector at equimolar ratio. The resulting vector was transformed into 10-beta competent Escherichia coli cells (New England Biolabs) and grown overnight on LB plates supplemented with 50 µg/mL ampicillin (Sigma-Aldrich). Colonies were pooled by flushing plates with LB supplemented with 50 µg/mL ampicillin. After overnight culture, plasmid DNA was extracted with the use of the GenElute HP Plasmid Midiprep Kit (Sigma-Aldrich).

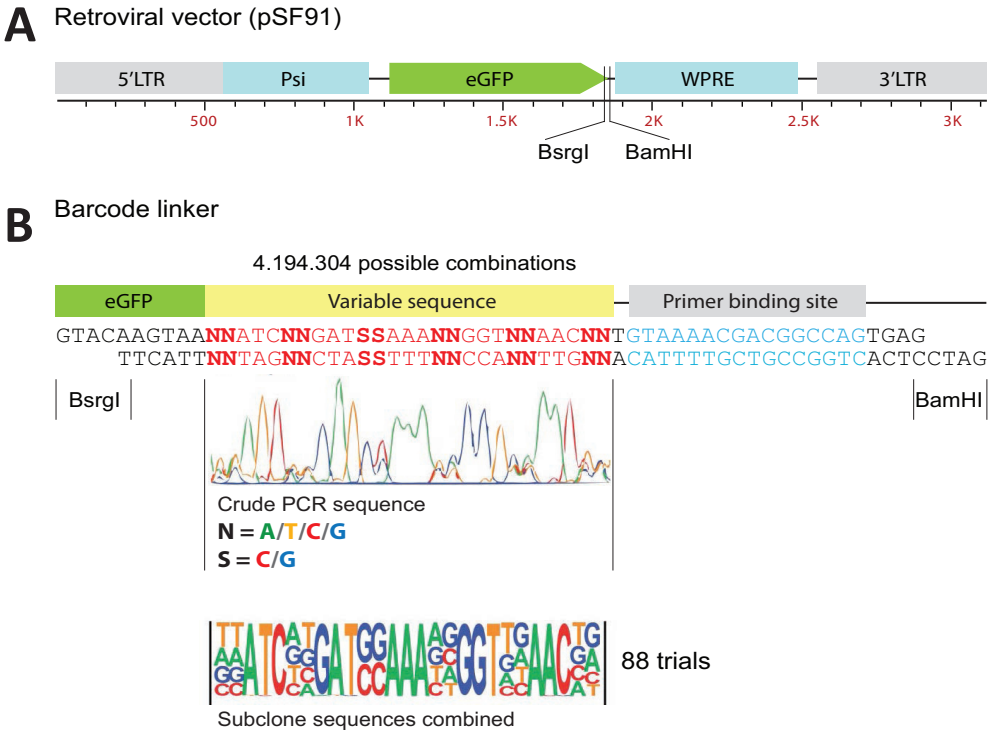


Figure 1. Construction and validation of barcoded vector library. A Insertion of the barcode linker into retroviral vectors SF91 and MIEV. The linker contains a variable sequence part that consists of pairs of degenerate nucleotides (N or S) flanked by fixed triplets. The crude PCR sequence trace of the resulting vector batch suggests the random insertion of nucleotides at N and S positions. **B** The vector library, created by combining approximately 800 bacterial clones, was retransformed into Escherichia coli. By combining the sequence traces of the 88 resulting clones, the crude PCR sequence trace could be reconstructed.

Validation of barcode vector library

Random sequence tags or 'barcodes' were generated by annealing 2 synthetic oligonucleotides consisting of sets of random nucleotides separated by fixed triplets [19]. The resulting double-stranded linker was then ligated into the retroviral vector (Figure 1A). Although in theory more than 4 million possible linker variants could be generated, in practice the number of variants was restricted to the number of bacterial clones generated on transformation. From each resulting vector library, barcode sequences were amplified with primers directed against internal vector sequences, after which the resulting mixture of equal-sized PCR products was sequenced. The resulting crude sequence traces suggested that a largely random mixture of sequence tags was generated, because N and S positions were found to be essentially equal in all 4 (A, T, C, and G) or 2 channels (C and G), respectively (Figure 1A). To validate the complexity of the prepared barcode libraries, we transformed a high complexity vector batch (created by combining 800 bacterial clones) back into E. coli and performed monoclonal sequence analysis. Of 88 sequencing trials, 78 represented unique barcodes. By combining in silico the sequence traces of all 88 trials the crude PCR sequence trace could be reconstructed (Figure 1B), confirming that the insertion of nucleotides at each variable position was close to random. Together, these data confirmed that the generated vector libraries are indeed composed of vectors marked with randomly generated barcodes.

Retroviral transduction of bone marrow cells

Post 5FU primary BM cells were transduced as previously described [19, 22-23]. Briefly, Phoenix ecotropic packaging cells were cultered (75cm^2 filter flasks, Greiner Bio-One) and transfected in 3.5-cm wells with 1 µg of barcoded vectors containing about 200-300 barcodes. Virus-containing supernatant harvested 24 and 48 hours later was used to transduce 7.5×10^5 BM cells per 3.5-cm well. Transduction efficiencies were determined by flow cytometry (BD, LSR-II) and varied between different experiments.

Bone marrow transplantation

Freshly transduced HSPCs (4.5-7.5 x10^6; CD45.1) were transplanted into lethally irradiated (9.0 Gy) CD45.2 mice, without prior GFP sorting. The relative contribution of transduced cells (GFP+) to different hematopoietic lineages in the blood, was determined by blood withdrawal from the retro-orbital plexus at indicated time points, as described previously [22].

At clear signs of morbidity, blood was taken from the orbital plexus. Sick mice, were euthanized and bone marrow cells were isolated by crushing femurs, tibiae and pelvic bones. Single-cell suspensions were prepared from the spleen. Blood, bone marrow and spleen cells were analyzed as described previously [22]. For serial transplantation experiments, 2-5x10^6 donor whole bone marrow cells were injected per recipient mouse.

Barcode recovery and identification

Genomic DNA was extracted from purified blood, bone marrow and spleen cells with the use of the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich) followed by a clean-up step using the Nucleospin Plasma XS kit (BioKe, Macherey-Nagel). Individual samples were amplified using unique multiplexing primers, containing a sample tag, directed against the internal EGFP vector sequence (eGFP forward) in combination with a uniform SF91 WPRE reverse primer (5'-CCCTAAAAATGTAAATGATTGCCCCACC-3'). 35-cycle amplification was performed using DreamTaq Green mastermix (Fermentas). Each barcode sample was amplified in duplicate and the mixture of equal-sized PCR products was pooled and purified (Roche, High Pure PCR Cleanup Micro Kit). Next, products were phosphorylated using polynucleotide kinase (Fermentas) at 37C for 30 minutes, after which an additional round of purification was performed. Sequencing was performed using an Illumina HiSeq2000 sequencer in the sequencing facility of University Medical Centre Groningen (using FASTQ format Illumina 1.3+).

In addition to deep sequencing, barcodes from gDNA from selected samples were amplified using eGFP forward 5'-CTGCCCGACAACCACTACCTG-3'; and SF91 WPRE reverse, 5'-CCCTAAAAATGTAAATGATTGCCCCACC-3') or LTR reverse 5'-GCCTTGCAAAATGGCGTTACTGC-3', individually purified, subcloned into pCR4-TOPO-TA (Invitrogen) and transformed into 10-beta competent E coli cells (New England Biolabs). Analyses of barcodes were performed using Sanger sequencing.

Deep sequencing data processing and noise filtering

Different sequence runs comprised up to approximately 200 samples per batch. Raw FASTQ sequence data underwent multiple rounds of filtering to remove low quality reads using custom-written scripts in Perl (v5.14.2), Bioconductor (2.11) in conjunction with R (2.15). Scripts are available upon request. All high quality sequences were compressed into a set of unique sequences, where frequencies of all unique sequences were counted. Sequences occurring only once (singletons) were removed.

Within the remaining reads, all identical reads were pooled and their frequencies were counted. Unique reads were split on the basis of exact match to 8-9nt of the sample tag of the forward primer and its 5' flanking sequence (13 nt total), resulting in a list of all reads that belong to one experimental sample (sample sets). Within each sample set the data were sorted in descending order on the basis of frequencies. Barcode sequences were extracted by calculating the position of the sample tag, primer length, and remaining distance to the first variable barcode position, using Python. This included the full length variable region of the barcode, plus the 5 base-long flanking sequences from both ends of the barcode. In each data set, barcodes were pairwise compared (in descending frequency order) for similarity using Levenshtein distance algorithm (Python-Levenshtein 0.10.2 package).

Infrequently occurring barcodes with a distance 0 or 1 were considered as sequence errors and not considered for analysis [21, 24]. In addition, samples for which in total less than 125 reads were recovered, were considered as failed and removed.

The top 100 unique sequences from each sample set were used to create a combined set (tables) of experimental data for each transplantation experiment. Within each experimental set sequences were checked for presence of barcode sequence, and only those that carried a recognizable barcode structure were taken into consideration. In each experimental dataset a 1% cut-off for cumulative barcode frequency was used: all barcodes exceeding this 1% threshold were taken for further analysis. The barcode that was most frequently found among all samples from one transplantation experiment was numbered barcode #1, the second most frequently appearing barcode was numbered barcode #2, etc.

Statistics

In case of multiple vector integration, a clone can be composed of multiple barcodes. In that case, two or more barcodes will be found in approximate equal ratios ('linked barcodes') in all analyzed samples within a specific transplantation experiment. The presence of linked barcodes due to multiple vectors integrating in a single cell was tested using a custom script in Python employing the Bray-Curtis spatial distance metric from Scipy (http://docs.scipy.org/doc/scipy/reference/spatial.distance.html). We tested whether fluctuations of barcodes in different samples from the same transplantation experiment occured by random using Monte Carlo modelling with maximal 30% fluctuations and an average dataset of 12. The expected frequency of multiple hits was calculated using Poisson distribution and provided as p-values. All linked barcodes with $p<0.05$ were considered as coming from the same clone.

RESULTS

Overexpression of Cbx7 in bone marrow induces different types of leukemia

Overexpression of the Polycomb member Cbx7 in post-5FU bone marrow cells (which include hematopoietic stem and progenitor cells; HSPCs) has been shown to induce multiple leukemia subtypes [22]. These leukemias were characterized by morphological and immunophenotypic analyses of cells isolated from various hematopoietic tissues such as blood, bone marrow, spleen and lymph nodes.

The majority of mice transplanted with Cbx7-overexpressing bone marrow cells developed a T-cell leukemia (Figure 2). In these mice, extensive extramedullary hematopoiesis was observed; white blood cell counts were increased, and all mice showed splenomegaly and enlarged lymph nodes.

Some mice showed hepatomegaly and thymic enlargement as well. FACS analysis showed that a large proportion of cells in these tissues expressed CD3ε, CD4, and CD127 [22].

A second type of leukemia was classified as an immature leukemia (Figure 2), since malignant cells did not express any of the lineage markers used for analysis (CD3ε, CD3, CD4, CD127, Ter119, Gr1, Mac1, B220, CD16/32, CD41). Extramedullary hematopoiesis was observed in the spleen, but not in lymph nodes. Peripheral white blood cell counts were increased, and cells in blood, bone marrow and spleen showed a primitive blast-like morphology [22].

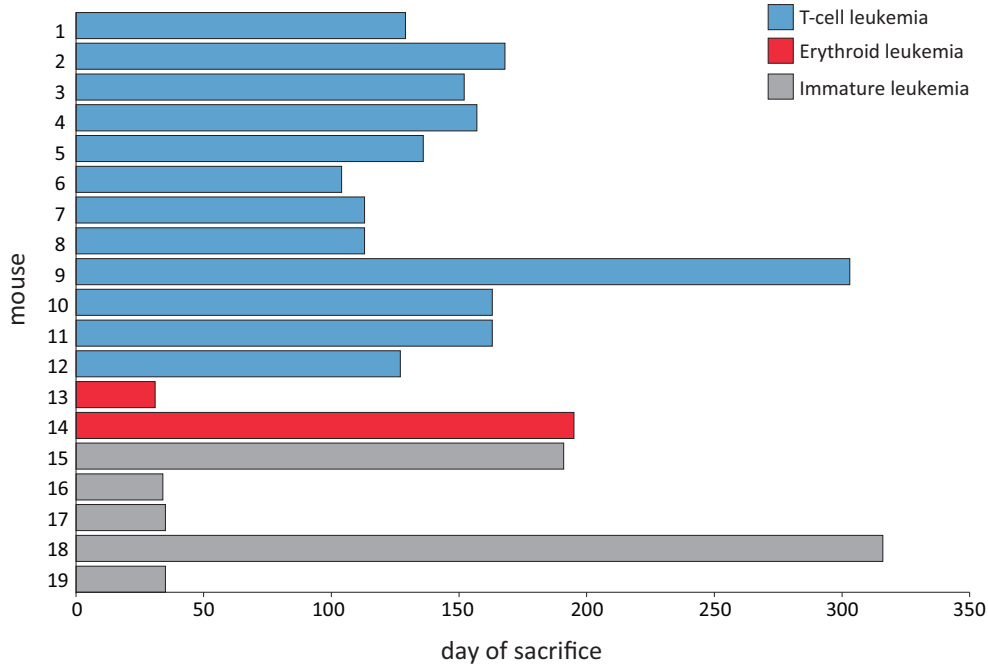


Figure 2. Cbx7-induced leukemia phenotypes. Post-5FU-treated bone marrow cells from CD45.1 mice were isolated and transduced with control or Cbx-overexpressing retroviral vectors. Freshly transduced cells were then transplanted into lethally irradiated CD45.2 mice, without prior sorting for GFP. Mice developed different types of leukemia, indicated by the color of the bar, at indicated timepoints (x-axis).

Finally, two mice developed an erythroid leukemia (Figure 2). In contrast to spleens of mice that developed an immature or T-cell leukemia, spleens of erythroid leukemic mice were only mildly enlarged and were colored dark red, instead of red with white patches reflecting areas of extramedullary hematopoiesis of white blood cells. Lymph nodes were not enlarged, but one mouse showed hemorrhagic lesions in lymph nodes. Peripheral white blood cell counts were decreased, and increased numbers of reticulocytes and decreased numbers of mature erythrocytes

were detected. In addition, microscopic evaluation of bone marrow and spleen samples revealed numerous erythroid precursors at variable stages of maturation. Malignant cells expressed Ter119 [22]. Together, overexpression of Cbx7 induces leukemias with a high penetrance but with variable phenotypes. Thus, Cbx7 has a strong, but dynamic oncogenic potential.

In this study, HSPCs were transduced with a Cbx7 vector library (or control vector library) composed of 200-300 unique barcoded vectors. After transduction, 4.5-7.5x10^6 non-sorted cells were transplanted in irradiated recipients. At selected timepoints after transplantation, cells were isolated from different hematopoietic tissues, analyzed and/or purified by flowcytometry, and barcodes were retrieved from genomic DNA using deep sequencing. In this model, overexpression of Cbx7 serves as the initial leukemic 'hit' and every pre-LSCs is uniquely labelled. This allows for the identification of single LSC–derived clones in the transplant recipient. Clonal waves of HSC contributions to the blood and emergence and persistence of clonal dominance was analyzed by regular blood sampling (week 4,8,16 and 24) (Figure 3, 1). The clonal composition in blood, bone marrow and spleen was analyzed after leukemia had developed in mice (Figure 3, 2). In several instances, bone marrow cells were isolated from leukemic mice and serially transplanted in secondary recipients (Figure 3, 3).

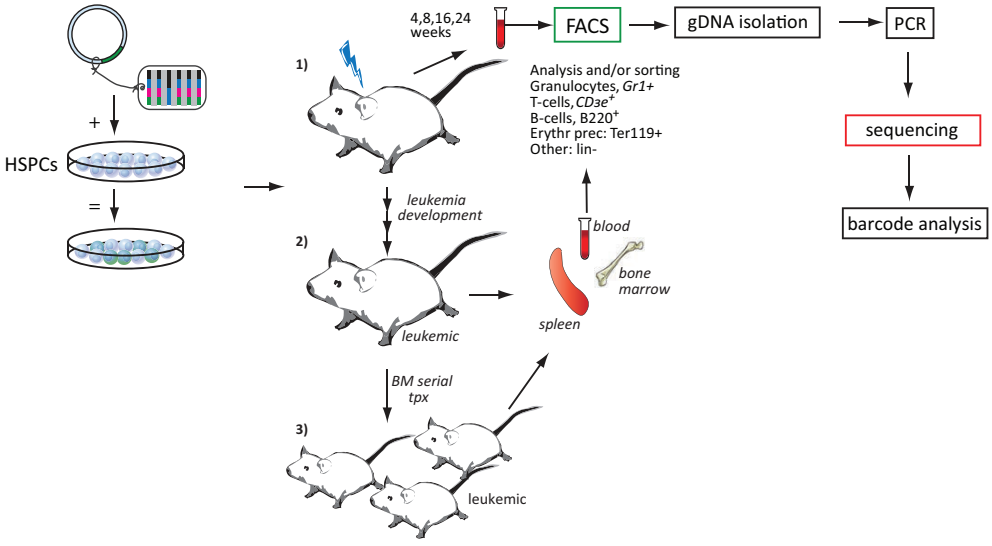


Figure 3. Schematic representation of experimental set-up. HSPCs were transduced with a barcoded Cbx7 vector library and transplanted in irradiated recipients. **1** Clonal contributions of HSCs to the blood was analyzed by regular blood sampling (week 4,8,16 and 24). **2** The clonal composition in blood, bone marrow and spleen was analyzed after leukemia development in mice. **3** Bone marrow cells were isolated from leukemic mice and serially transplanted in secondary recipients. For clonal analysis, cells were analyzed and/or purified by flowcytometry, and barcodes were retrieved from gDNA using deep sequencing.

Cbx7-induced leukemias are not obligatory monoclonal

We analyzed contributions of major and minor clones in different hematopoietic tissues in every individual control or leukemic mouse. To this end, we retrieved barcodes from blood, bone marrow and spleen samples taken at the time of sacrifice. In some cases, multiple vectors may have integrated in a single cell. In these cases, one barcode does not reflect one single clone, but a clone can be composed of multiple barcodes. Indeed, using Bray-Curtis distance algorithm (see Methods), we have found dominant but also minor clones with multiple vector integrations in some leukemic mice (for example, Figure 4d mouse 4: barcode 1a, 1b, and 1c, represent clone #1, Figure 4f mouse 16: barcode 1a-1d represent clone #1, mouse 17, barcode 1a-1d represent clone #1, Supplementary Figure S2a-b). These mice were transplanted with bone marrow cells where a 40% transduction efficiency was reached. Since the random occurrence of 3-4 integration sites under these conditions is still small (~2% of cells. Poisson, $p<0.05$, [24]), this indicates that Cbx7 gene dosage due to multiple vector integrations might have a positive effect on cell proliferation and clone selection.

As expected, all hematopoietic lineages in control, non-leukemic mice were repopulated by bone marrow cells transduced with barcoded retroviral vectors (Figure 4a). The hematopoietic system of most of these mice was repopulated by 13-26 different clones (Figure 4b, mouse 1 and 2, Figure S1 mouse 4 and 5). Typically, 2-3 major clones were found to predominate in all different hematopoietic tissues (blood, bone marrow and spleen) and made up approximately 30%-60% of all cells. One mouse displayed a biclonal hematopoietic system (blood, bone marrow and spleen) (Figure 4b, mouse 3), but this did not evolve in malignancy. We suspect that this mouse was transplanted with a low number of stem cells.

In Figure 4c-f we display the cell lineages and extent of clonality in various hematopoietic tissues (blood, bone marrow and spleen) of mice transplanted with bone marrow cells transduced with barcoded Cbx7-expression vectors that all developed leukemia. In general, the hematopoietic system of Cbx7-induced leukemic mice (Figure 4c-f, Figure S2a-b) was composed of relatively fewer clones compared to healthy controls (Figure 4b, Figure S1). However, surprisingly, in many cases the leukemic hematopoietic system did not show monoclonality (Figure 4c-f, Figure S2a-b). Although some T-cell leukemias were clearly monoclonal (Figure 4c, Figure S2a) and in these cases one clone constituted 80-100% of all cells in the blood, bone marrow and spleen, other mice, with a phenotypically identical disease, displayed oligoclonal T-cell leukemias (Figure 4d, Figure S2a). In these mice, two to four LSC clones simultaneously contributed to malignant outgrowth of T-cells in the blood, bone marrow and spleen, resulting in increased white blood cell counts and splenic enlargement.

Mouse 3 (Figure 4d), presented with a large number of CD3ε+ T-cells, but also the number of $Ter119^+$ erythroid precursors and immature hematopoietic cells (Lin-) was excessive in blood, bone

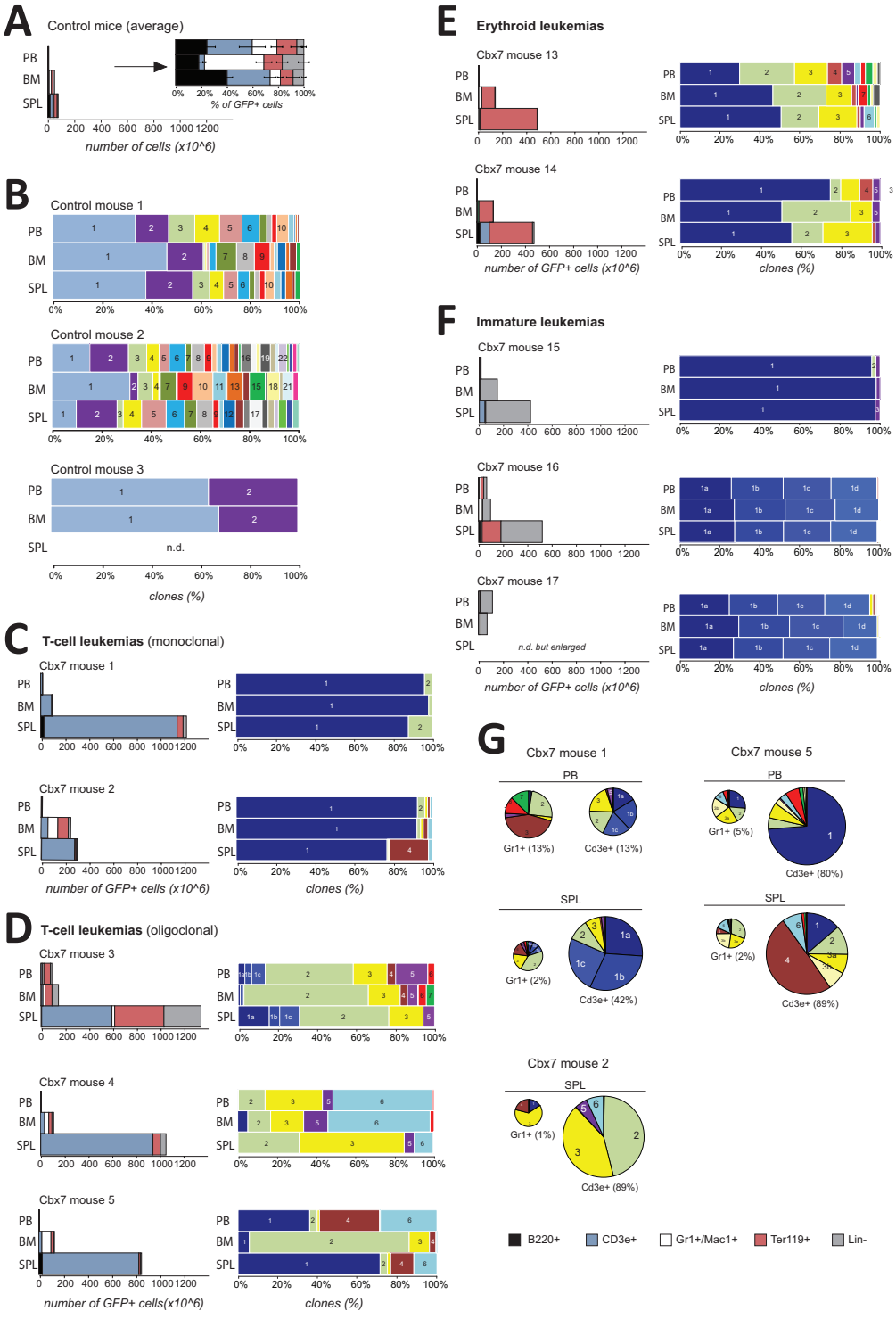

A
Control mice (average)
PB
BM
SPL
% of GFP+ cells
number of cells (x10^6)
B
Control mouse 1
Control mouse 2
Control mouse 3
n.d.
clones (%)
C
T-cell leukemias (monoclonal)
Cbx7 mouse 1
Cbx7 mouse 2
number of GFP+ cells (x10^6)
clones (%)
D
T-cell leukemias (oligoclonal)
Cbx7 mouse 3
Cbx7 mouse 4
Cbx7 mouse 5
number of GFP+ cells(x10^6)
clones (%)
E
Erythroid leukemias
Cbx7 mouse 13
Cbx7 mouse 14
number of GFP+ cells (x10^6)
clones (%)
F
Immature leukemias
Cbx7 mouse 15
Cbx7 mouse 16
Cbx7 mouse 17
n.d. but enlarged
number of GFP+ cells (x10^6)
clones (%)
G
Cbx7 mouse 1
Gr1+ (13%)
Cd3e+ (13%)
Gr1+ (2%)
Cd3e+ (42%)
Cbx7 mouse 5
Gr1+ (5%)
Cd3e+ (80%)
Gr1+ (2%)
Cd3e+ (89%)
Cbx7 mouse 2
Gr1+ (1%)
Cd3e+ (89%)
B220+
CD3e+
Gr1+/Mac1+
Ter119+
Lin-

Figure 4. Clonality in control and Cbx7-induced leukemic mic. A Average cell counts and percentage of different celltypes in blood, bone marrow and spleen of control mice (n=5, also see figure S1). **B** Control mice were transplanted with barcoded control vector transduced cells. The number of clones and their relative contribution to blood, bone marrow and spleen was analyzed. **C-F** Mice were transplanted with bone marrow cells transduced with barcoded Cbx7-expression vectors. The absolute contribution of different cell lineages in blood, bone marrow and spleen (left) and the contribution of different clones to the blood, bone marrow and spleen (right) in T-cell leukemic mice **C-D**, erythroid leukemic mice **E** and mice with an immature leukemia **F**. **G** Different clones in different hematopoietic lineages from the blood and the spleen. Different major and minor (as illustrated by the size of the pie) hematopoietic cell populations (Gr1+ granulocytes and CD3ε+ T-cell) were FACS-purified on basis of marker expression, gDNA was isolated and barcodes were extracted. Similar but also different barcodes contribute to different lineages. The size of the pie illustrates the percentage of cells in the indicated tissue.

Different clones in individual mice are indicated by different colors and their corresponding number. In all Cbx7 mice, the barcode that was most frequently found among all samples from one transplantation experiment was numbered barcode 1 (blue), the second most frequently appearing barcode was numbered barcode 2 (green) etc. Note that the clone numbering and coloring relates only to clones found in an individual mouse; similarly colored and numbered clones in separate mice refer to distinct clones.

marrow and spleen. Three distinct clones (blue clone 1 (with three vector integrations: barcodes a,b,c), green clone 2, and yellow clone 3) were likely collectively responsible for this malignancy. There are two explanations for such oligoclonal contributions to phenotypically distinct expanded hematopoietic celltypes. First, LSCs may have retained the ability to (an extent of) multilineage differentiation. Second, if instead LSCs have only unilineage differentiaton capacities, different LSC clones must have contributed to the expansion of different lineages. We cannot formally discriminate between these possibilities, since not all distinct cell populations have been separately analyzed for their clonal composition. However, since all three LSC clones contributed to malignant Cd3ε$^+$ T-cells (Figure 4g), and since the prevalence of these clones together is ~95% in the spleen, these very same clones most likely contributed to expanded erythroid and immature cells as well. This suggests multilineage differentiaton capacities of LSCs.

Mouse 4 and 5 (Figure 4c) both showed an oligoclonal T-cell leukemia. In mouse 4, the expansion of malignant CD3ε$^+$ cells is most profound in the spleen. In this mouse, clone 2 and 3 together constitute ~85% of cells in the spleen and the same clones are predominant in sorted CD3ε$^+$ cells (Figure 4g). In mouse 5, three clones (clone 1, 4 and 6) are responsible for the expansion of malignant CD3ε$^+$ cells (Figure 4c, Figure 5 mouse 5). In this mouse, but also in mouse 11 (Figure S2a), malignant clones predominated the blood and spleen, but not the bone marrow, in which no signs of leukemia were detected. This might suggest migration of malignant LSCs and/or their progeny to the blood and spleen, after which relatively normal hematopoiesis persisted in the bone marrow by the activity of other HSC clones.

The hematopoietic system of the two mice that developed an erythroid leukemia (mouse 13 and 14, Figure 4e) showed oligoclonality in blood, bone marrow and spleen. In contrast, the hematopoietic system of mice that developed an immature leukemia was always monoclonal (Figure 4f, Figure

S2b). We also analyzed the clonal composition of different hematopoietic cell populations in individual mice. To this end, different cell lineages from the blood and spleen were FACS purified and barcodes were retrieved from genomic DNA using deep sequencing (Figure 4g, Figure S2c-d). We found that minor non-malignant clones could still contribute to residual normal myelopoiesis (Gr1+ cells) in leukemic mice. In addition, dominant barcodes could mark both malignant T-cells as well as non-malignant granulocytes (Figure 4g, Figure S2c-d). Thus, the cell from which the leukemia derived possessed both lymphoid and myeloid differentiation capacities, which suggests that the oncogenic origin of leukemias lies within multipotent stem cells.

Together, our data suggest a distinct degree of competition between malignant clones in different types of leukmia that most probably originated from multipotent cells: we show the coexistence of multiple collaborating, or at least equally competitive, clones in erythroid leukemias and some T-cell leukemias, while clones in other T-cell leukemias and all undifferentiated leukemias were aggressively competing and ultimately resulted in one dominant clone.

Variable onset of clonal dominance

Phenotypical analysis and deep sequencing of barcodes from blood samples taken during the course of the transplantation experiment, allowed us to assess the timing of clonal dominance and leukemia development.

At early time points after transplantation, only a few clones (2-8 clones) contributed to blood cell regeneration of mice transplanted with Cbx7-overexpressing bone marrow cells (Figure 5, Figure S3). Some clones were already contributing to hematopoietic reconstitution early after transplantation, and showed multilineage differentiation capacities (for example Figure 5, mouse 1 clone 1, mouse 15 clone 1). These clones then expanded over time, to ultimately dominate the hematopoietic system (Figure 5, mouse 1 clone 1, mouse 15 clone 1, Figure S3 mouse 8 clone 1, mouse 14 clone 1). Although these clones initially showed multilineage differentiation capacities in the pre-leukemic period, at the timepoint of leukemia development, they showed malignant proliferation towards preferentially one hematopoietic cell type.

However, we also found cases in which minor clones, which remained undetectable in the blood at earlier timepoints, became dominant and contributed to leukemia development (Figure 5, mouse 5 clone 6, mouse 18 clone 1, Figure S3 mouse 11 clone 1). Together, our data reveal a highly variable time of onset of clonal dominance.

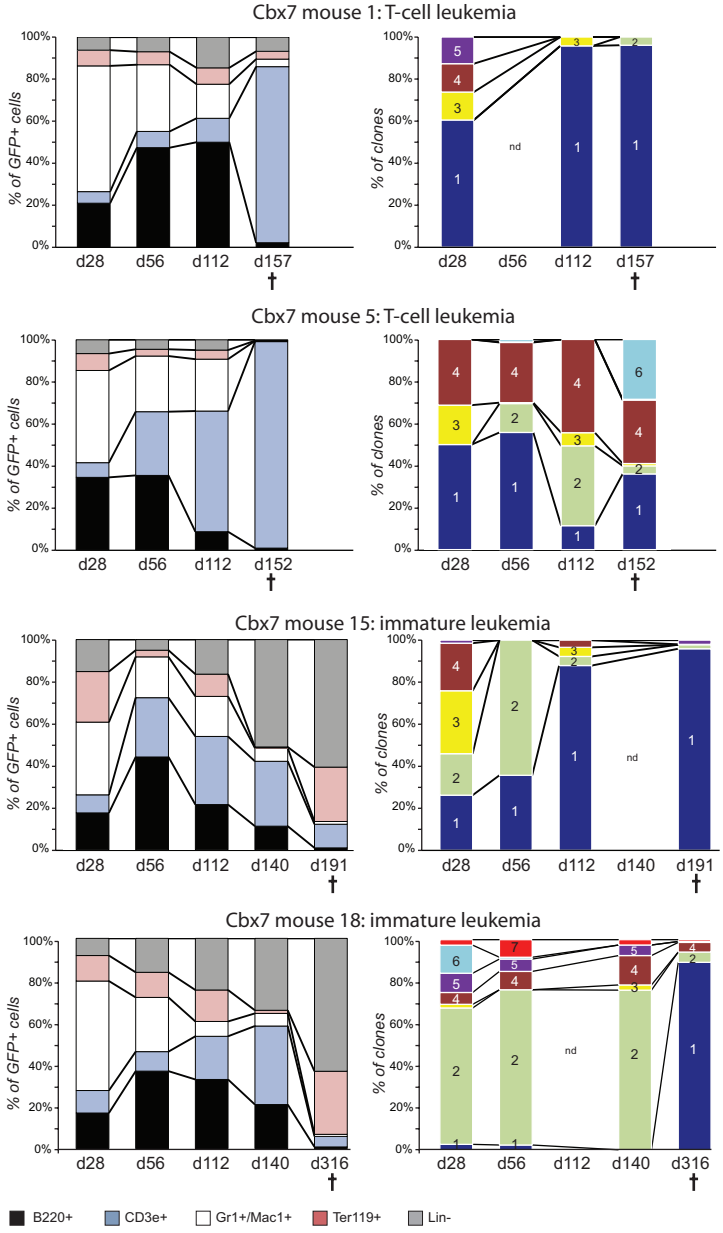


Figure 5. Onset of clonal dominance in Cbx7-induced leukemic mice. Left: percentage of different donor-derived GFP+ hematopoietic cell types in blood on indicated timepoints, as analysed by FACS. Right: The contribution of different clones to the blood on indicated timepoints. The final timepoint is the day of sacrifice at clear signs of morbidity. Different clones in individual mice are indicated by different colors and their corresponding number. In all mice, the barcode that was most frequently found among all samples from one transplantation experiment was numbered barcode 1 (blue), the second most frequently appearing barcode was numbered barcode 2 (green) etc. Note that the clone numbering and coloring relates only to clones found in an individual mouse; similarly colored and numbered clones in separate mice refer to distinct clones.

Clonal dynamics after serial transplantation

Heritability of clonal dominance and leukemia phenotype

Next, we tested whether Cbx7-induced leukemias were transplantable and whether the leukemic types as assessed by organ involvement, tissue morphology, and immune-phenotype were similar in the original donor and in secondary recipients. For this purpose, we serially transplanted bone marrow cells from five leukemic mice into 3 to 5 secondary recipients. Barcode analysis allowed us to determine whether the clonal origin of leukemia in secondary recipient mice was identical to the disease-causing clone in the original donor mouse.

In the first serial transplantation experiment, we observed a very stable pattern of disease phenotype and clonal dominance (Figure 6). Three secondary recipient mice transplanted with bone marrow cells from a primary recipient with a monoclonal T-cell leukemia, all developed T-cell leukemias as well. Recipients, like the donor, showed enlarged spleens with white patches (illustrating areas of extramedullary hematopoieis), enlarged lymph nodes (data not shown), and expansion of CD3e+ cells (Figure 6). Barcode analyses revealed that the disease-causing clone was identical in the donor mouse and all individual recipients (Figure 6, mouse 4 donor, and recipient 1-3, clone 1).

Activation of dormant LCS clones

Second, we observed the appearance of different leukemic phenotypes, coinciding with the emergence of new dominant clones after serial transplantation (Figure 7a,b). Bone marrow cells from donor mouse 2, with an oligoclonal T-cell leukemia were serially transplanted in 3 recipient mice, of which recipient 1 and recipient 2 also developed a T-cell leukemia (Figure 7a, recipient 1 and 2). In contrast, recipient 3 developed an immature leukemia (Figure 7a, recipient 3). In this mouse, malignant GFP+ cells in the blood, bone marrow and spleen did not express any of the lineage markers used for immunophenotyping, while the large majority of cells in mice which succumbed to a T-cell leukemia expressed CD3+. To understand the clonal dynamics associated with the appearance of different leukemic phenotypes after serial transplantation, different cell populations were FACS-purified from the blood and spleen of secondary recipients, and the contribution of each clone to different cell lineages was determined. Clone 2 and 3 were identified as the malignant clones present in the donor mouse, since these cells contributed to the expansion of CD3+ cells primarily in the spleen (Figure 7a, Figure 4g mouse 2). The same two clones were also highly dominant in expanded CD3+ cells in blood (68% and 95% of total cells) and spleen (91% and 95% of total cells) from recipient 1 and 2 that developed T-cell leukemia, similar to the donor. However, the immature leukemia in recipient 3 appeared to be of different clonal origin. Different clones (clone 1 and clone 4) were responsible for the expansion of immature cells in recipient 3, which composed

96% of cells in the blood and 98% of cells in the spleen. Interestingly, clone 1 and clone 4 also contributed to a modest expansion of immature cells in the spleen of recipient 1 (30% of total cells). These clones were barely detectable in the hematopoietic system of the donor mouse at leukemia diagnosis, and thus previously dormant.

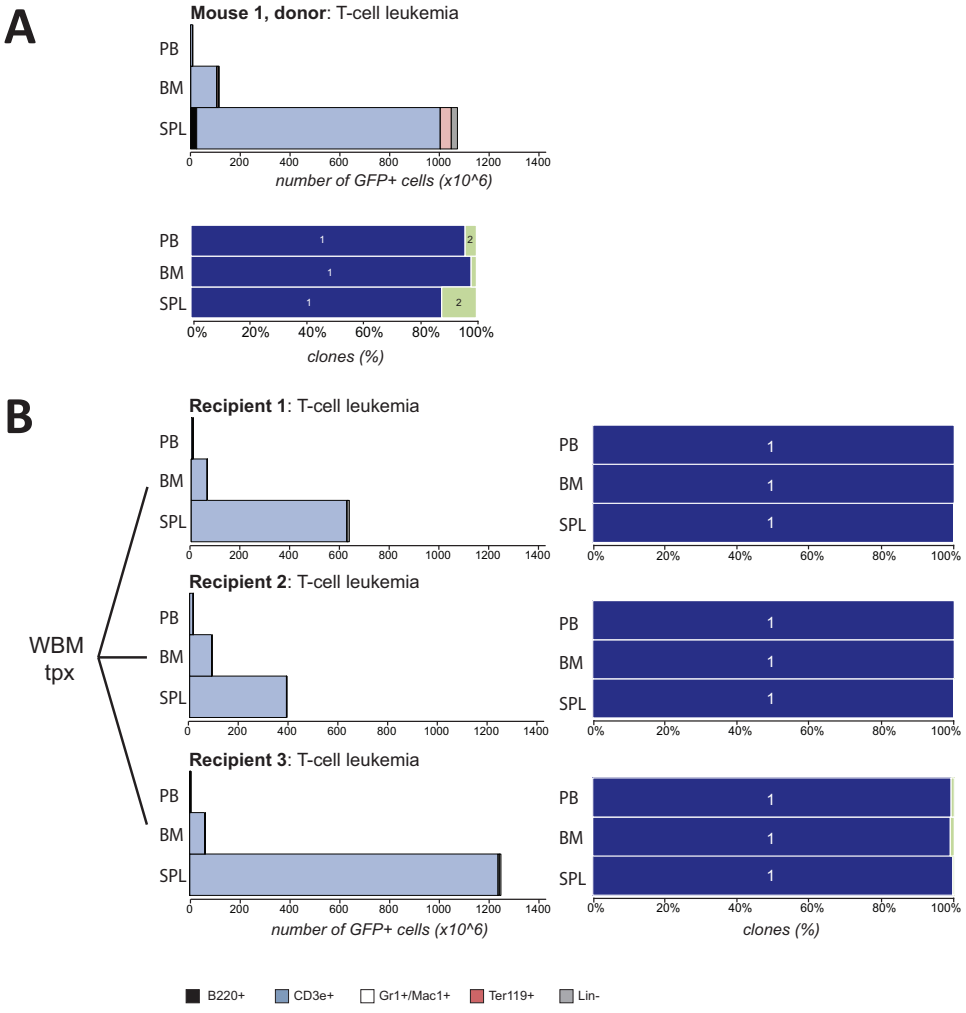


Figure 6. Heritability of clonal dominance and disease phenotype. Serial transplantation of bone marrow cells isolated from a mouse with a T-cell leukemia into 3 secondary recipients. **A** Absolute number of indicated cell types in blood, bone marrow and spleen (upper panel), and the clonal composition of blood, bone marrow and spleen (lower panel) in the donor. **B** Left panel: absolute number of indicated cell types in blood, bone marrow and spleen of secondary recipients transplanted with bone marrow cells from mouse 1. Right panel: The contribution of different clones to the blood, bone marrow and spleen of secondary recipients. Different colors represent different clones and are indicated by different numbers.

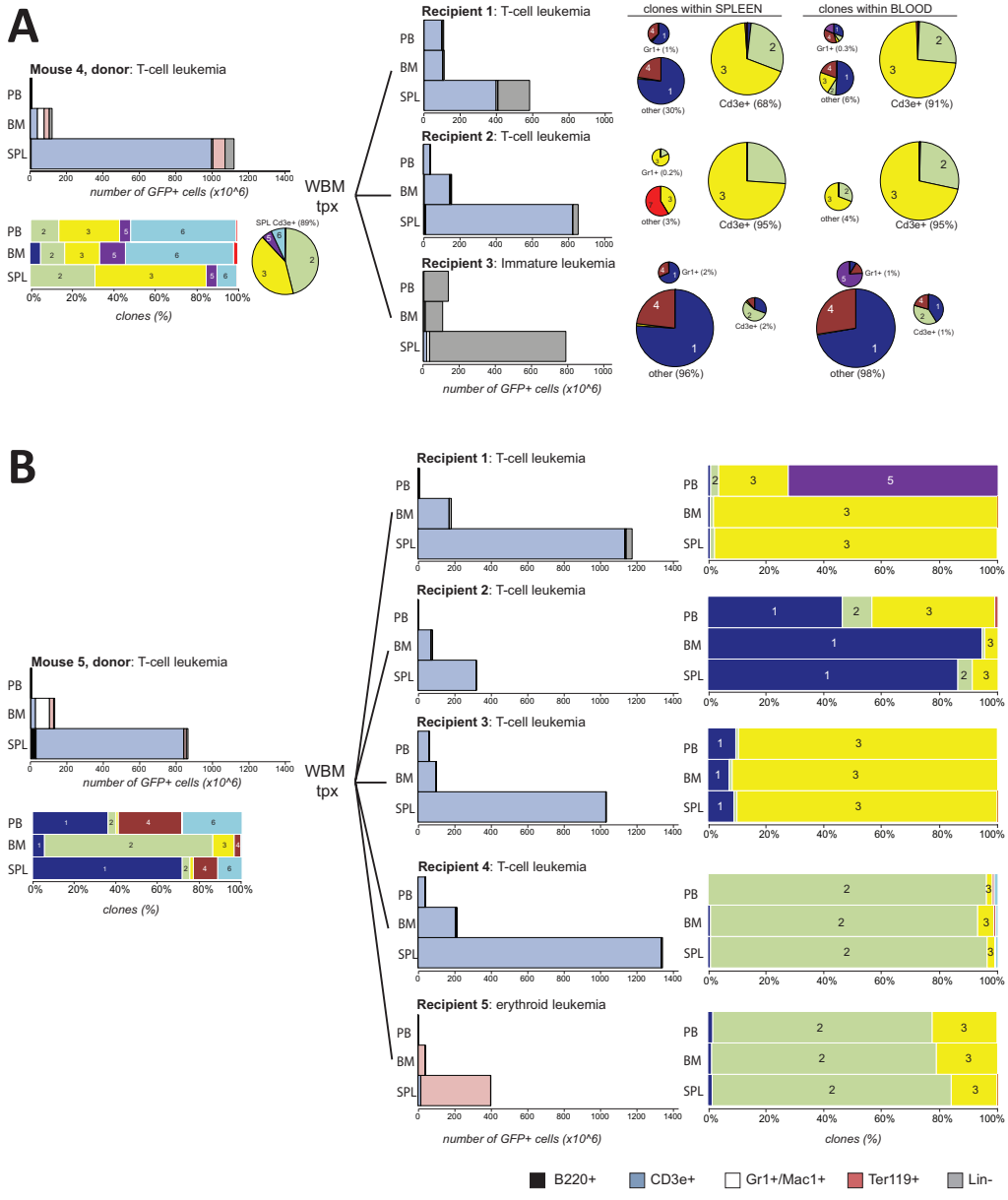


Figure 7. Activation of dormant LCS clones after serial transplantation

A Serial transplantation of bone marrow cells from a mouse with a T-cell leukemia into 3 secondary recipients. Recipients developed either a T-cell leukemia or a leukemia with an immature phenotype. Left: absolute number of indicated cell types in blood, bone marrow and spleen (upper panel), and the contribution of different clones in the blood, bone marrow and spleen (lower panel) in the donor. Right: Left panels: absolute number of indicated cell types in blood, bone marrow and spleen of recipients. Right panel: clonal composition of blood and spleen of recipients in sorted hematopoietic populations (Gr1$^+$ granulocytes, CD3ε$^+$ T-cells, or immature Lin- cells) of indicated mice. Size of the

pies reflect the percentage of cells in the indicated tissue. Different colors represent different clones and are indicated by different numbers. **B** Serial transplantation of bone marrow cells from a mouse with a T-cell leukemia into 4 secondary recipients. Recipients develop either a T-cell leukemia or an erythroid leukemia. Left: absolute number of indicated cell types in blood, bone marrow and spleen (upper panel), and the contribution of different clones to the blood, bone marrow and spleen (lower panel) in the donor.

A similar pattern of activation of minor clones was observed in the third serial transplantation experiment (Figure 7b). While in the donor mouse, clone 1, 4 and 6 drove T-cell malignancy predominantly in the blood and spleen (Figure 7b, figure 3c mouse 3), in recipient mice different novel clones were contributing to leukemia (Figure 7b). Only recipient 2 inherited the same dominant clone (clone 1, blue) as one of the disease causing clones in the donor, driving a similar disease phenotype (T-ALL). Clone 3 (yellow), which was relatively minor in the donor mouse, became highly dominant after serial transplantation, causing T-cell leukemia in recipient 1 and 3. Although clone 2 (green) was highly prevalent in the bone marrow of the donor mouse, at the time of sacrifice it was not yet malignant since it did not contribute to the expansion of T-cells in blood and spleen. However, this clone developed into a T-cell leukemia in recipient 4. Surprisingly, the very same clone caused development of an erythroid leukemia in recipient 5. In another experiment (data not shown), serial transplantation of bone marrow cell from a mouse with a T-cell leukemia also resulted in development of an erythroid leukemia in one of the secondary recipients. While all recipients with a T-cell leukemia showed enlarged spleens with white patches, containing areas of extramedullary hematopoiesis of white blood cells, enlarged lymph nodes, and expression of $CD3\varepsilon^+$ on GFP^+ cells, the spleen of recipient 5 was colored dark red, lymph nodes were not enlarged, and cells expressed $Ter119^+$ (Figure 7b, Figure S4). The observation that a single clone can induce two morphologically and immunophenotypically distinct types of leukemia documents that this particular clone retained multilineage differentiation capacities.

In these two serial transplantation experiments, we document the existence of minor or dormant clones with leukemic potential in the donor mouse. Upon serial transplantation of bone marrow cells, these clones can cause leukemia in secondary recipients with a similar or different disease-phenotype.

Differentiation of LSC clones after serial transplantation

Third, we observed evolution of leukemic clones from an immature phenotype to the lymphoid lineage. Bone marrow cells from a mouse with an immature leukemia, which did not express any of the immunophenotypic lineage markers (mouse 15, Figure 8), were serially transplanted into three recipient mice. Strikingly, all three recipients (recipient 1, 2, 3) developed a similar $CD3\varepsilon^+$ T-cell leukemia. Barcode analysis of the blood, bone marrow and spleen showed that a single clone (clone 1) is highly dominant in the donor as well as

in all recipients. Thus, leukemias in the recipients originate from the same LSC clone as the leukemia in the donor. However, differentiation of the LSC clone into the lymphoid lineage after serial transplantation, resulted in a phenotypically distinct leukemia type in all recipients. Thus, phenotypically distinct leukemias might have similar clonal origins.

Altogether, these observations show that different clones with different leukemic properties can coexist in a single mouse. Minor LSC clones with similar or different differentiation potential can be relatively dormant in the bone marrow of a leukemic mouse and progress to full-blown leukemia only after serial transplantation. In addition, some LSCs clones retain multilineage differentiation capacities and one LSC clone can therefore induce phenotypically distinct leukemias.

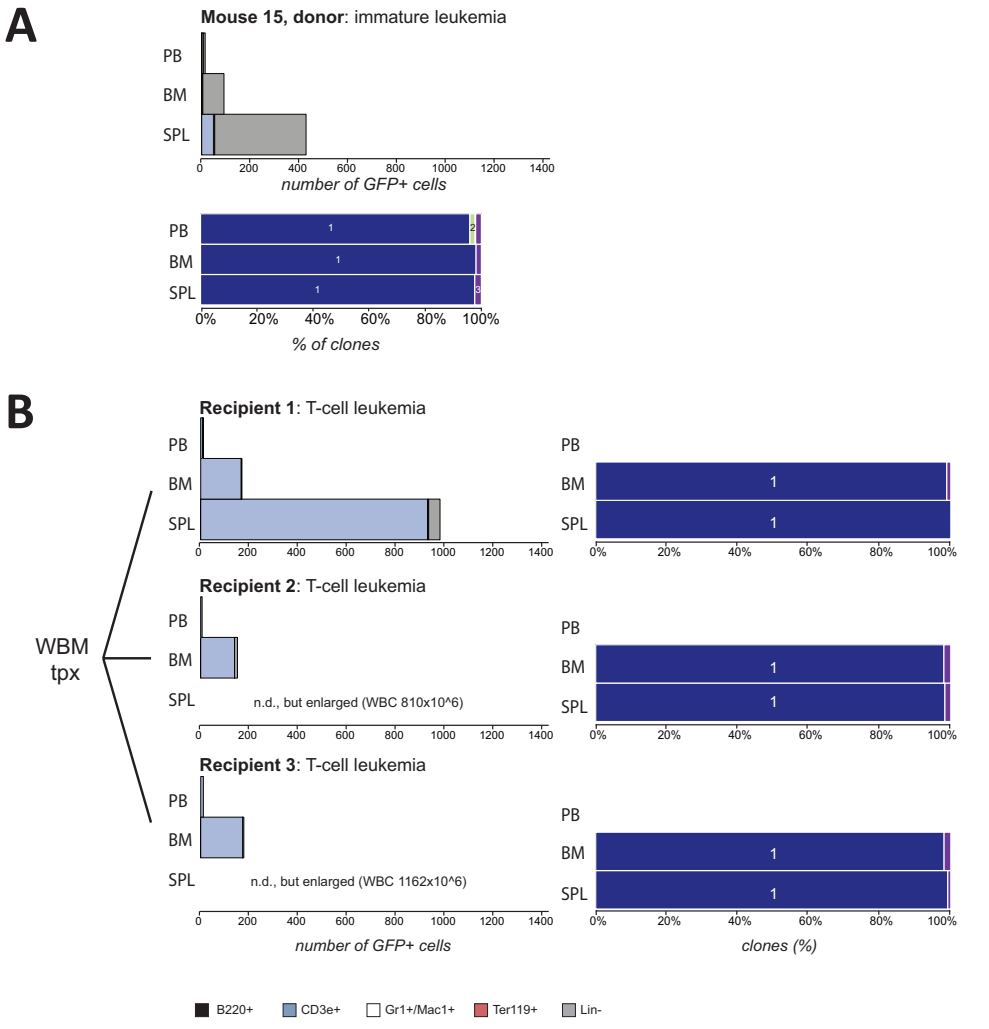


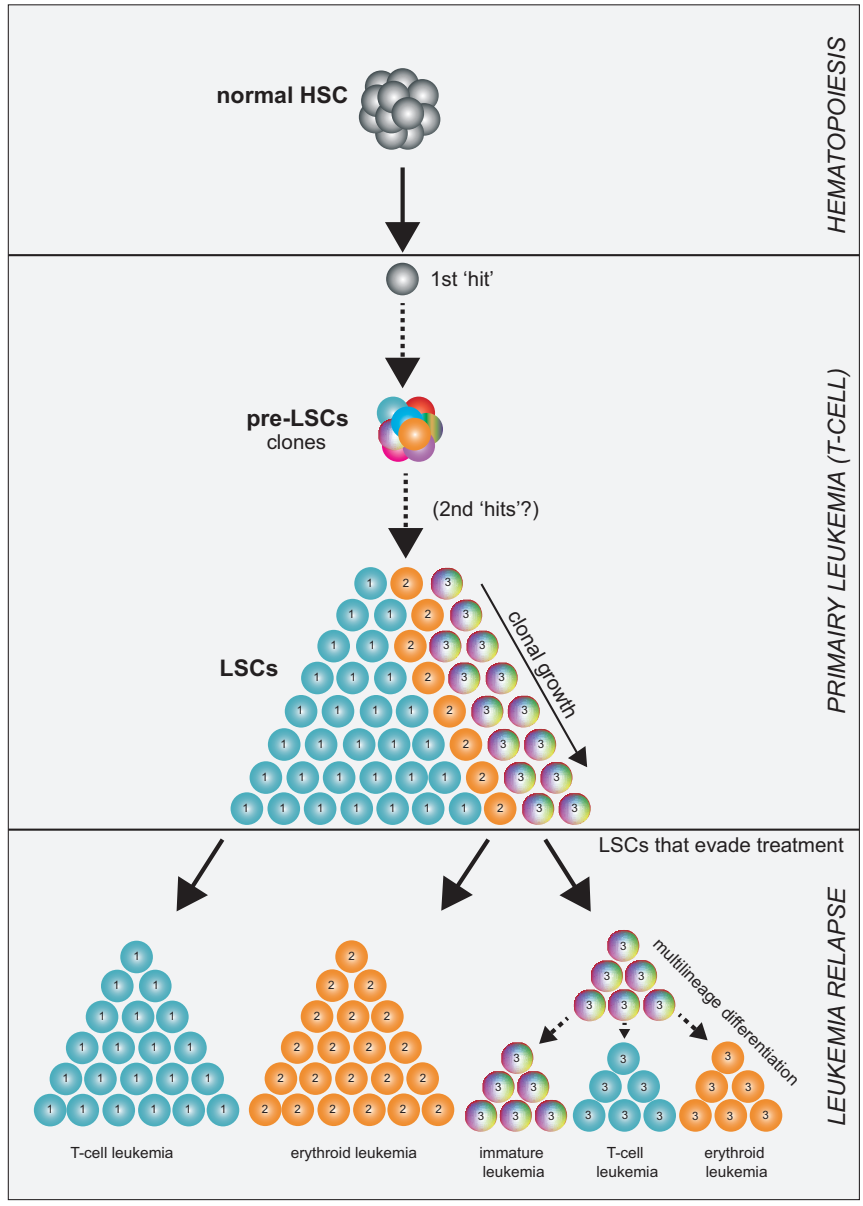
Figure 8. Differentiation of LSC clones. Serial transplantation of bone marrow cells from a mouse with an immature leukemia into 3 recipients. Recipients all developed a T-cell leukemia. **A** Absolute number of indicated cell types in blood, bone marrow and spleen (upper panel), and the clonal composition of blood, bone marrow and spleen (lower panel) in the donor. **B** Left panel: absolute number of indicated cell types in blood, bone marrow and spleen of recipients. Right panel: The contribution of different clones to the blood, bone marrow and spleen. Different colors represent different clones and are indicated by different numbers.

DISCUSSION

In early conceptual tumor models, malignant cells were thought to descend from a single common ancestor whose offspring sequentially acquired multiple mutations or other genetic abnormalities in time [1-3]. This model implies that all cells in a tumor are linearly related to each other and are homogenous in their mutational landscape and behavior. In contrast, more recent studies suggest that at least some tumors display substantial phenotypic and genetic heterogeneity, whereby cellular evolution can result in a branched model of tumor development [5-6, 12-15, 25, 26].

Although emerging evidence suggests that leukemias (and other tumors for that matter) are comprised of a multitude of different subclones, our understanding of the dynamics of tumor clonality remain very limited. Also, preclinical mouse models of leukemia have so far not employed *in vivo* tracking of clonal progression and leukemia transformation. In the current study, we pioneered a novel barcoding tool combined with overexpression of Cbx7, an epigenetic modifier capable of inducing self-renewal of HSCs [22], to discern patterns of clonality in highly variable leukemic subtypes. We delineate leukemic heterogeneity with unprecedented resolution, and document the coexistence of (sometimes quiescent) LSCs with different leukemic properties in one tumor.

Our analysis revealed three general patterns of clonal evolution (figure 9). First, some leukemic clones are highly stable and dominant. These clones rapidly and strongly dominate the hematopoietic system of the primary donor mouse, and upon serial transplantation cause a similar type of leukemia in secondary recipients. A second pattern consists of minor clones in primary donors that become activated and highly dominant after serial transplantation. Leukemias in these recipient mice are thus of different clonal origin than the disease-causing dominant clone in the original donor mouse. As a consequence, the leukemic type (lymphoid, erythroid, or immature) in the recipients can be either similar or different as observed in the primary donor. Third, we have observed evolution (or differentiation) of leukemic clones from immature to the lymphoid lineage. In these cases, the leukemias in the donor and the recipients are of the same clonal origin, but in the recipients the leukemia manifests as a different clinical subtype. In all three cases we show that clonal dominance is not a prerequisite for end-stage leukemia.



LSC with T-cell differentiation properties

LSC with erythroid differentiation properties

clone with multilineage differentiation capacities

1,2,3 clone number

Figure 9. A model of clonal contributions to leukemia development and relapse. Our study indicates that after a first (epigenetic) hit, normal hematopoietic stem cells can evolve in pre-LSCs resulting in clones with different properties. LSC clones can either agressively compete and induce monoclonal leukemias, or rather coexist and produce oligoclonal leukemias, as depicted in this figure. Distinct LSC clones can have different properties, resulting in different disease phenotypes at relapse. Quiescent LSCs can become activated, resulting in phenotypically different disease at relapse (clone '2'). If leukemia arises from a LSC with multilineage potential (clone '3'), a relapsed leukemia can be of similar or different phenotype. Secondary epigenetic or genetic mutations can potentially further drive tumor heterogeneity.

Several severely diseased mice displayed an oligoclonal leukemia, but in addition, minor clones can be present in the bone marrow of a leukemic mouse and progress to leukemia only after serial transplantation. Secondary epigenetic or genetic mutations can potentially drive clonal evolution and tumor heterogeneity.

The heterogeneity in differentiation capacities of LSC clones supports the hypothesis that in our model the origin of LSCs resides within multipotent stem cells. First, we show that LSC clones in a single mouse can contribute to malignant proliferation of cells of different lineages (e.g. mouse 3 Figure 4). Second, we found that dominant barcodes can mark both malignant T-cells as well as healthy granulocytes. Third, cells belonging to a single clone can display different leukemic phenotypes upon serial transplantation. Togvether, this indicates that the leukemia-initiating stem cell has retained multilineage differentiation capacities. Our data do not allow to directly estimate the frequency with which LSCs occur in leukemic mice. However, the observation that ample disease heterogeneity was induced among secondary recpients, suggests that LSCs within these grafts were present at limiting dilution.

We show that Cbx7 can induce different types of leukemia [22]. It remains to be determined how one apparent genetic abnormality (Cbx7 overexpression) can induce three distinct disease phenotypes. Since Cbx7 is an epigenetic modifier, it is tempting to speculate that overexpression of Cbx7 generates an altered chromatin structure that is susceptible to stochastic epigenetic or genetic secondary 'hits', which ultimately shape the disease phenotype. There is precedent in cancer biology for a single genetic abnormality to cause different diseases, for example, a recurrent mutation in Jak2 (V617F) can cause three types of chronic leukemias, known as Essential Thrombocythemia, Polycythemia Vera and Myelofibrosis [27-29].

The dynamic clonal behavior, and conversion of leukemic phenotype that we observed in our mouse model shows resemblance with patients presenting with leukemic relapse after treatment. First, if remissions in relapsed patients are treated similarly as the original leukemia, the response is often different, indicating that emerging clones have novel properties [30-32]. Second, lineage conversions in relapsed leukemia patients have been

reported recurrently. A conversion from ALL to AML is most common, particular in pediatric patients [33-39]. Conversions from AML to ALL occur less frequently, but have been reported for both children and adults [39-46]. Case reports of other types of leukemic conversions, such as erythroleukemia into ALL have also been published [47]. In leukemic patients showing a relapse of a different lineage (so-called 'lineage switch'), leukemic clones often have a different morphology, cell size, amount of cytoplasm, presence of Auer rods and new phenotypic lineage markers 47 37. These clinical data are highly reminiscent to our current findings. By identifying the cell of origin of the initial leukemia (in the donor) and the secondary (relapsed) leukemia (in the recipient), we show that phenotypically distinct secondary leukemias can originate both from the same LSC clone that retained multilineage differentiation capacities, or from a different LSC clone with different leukemic properties.

Overall, we conclude that the clonal make-up of leukemias can be more complex than previously anticipated. We provide direct evidence for the quiescent nature of LSCs and show lineage conversion at the clonal level. Leukemias can be oligoclonal, with clone-dependent variability in multiple biological traits. As not all clones are equal, therapies should focus on eliminating all clones with variable behaviors, including quiescent LSCs. As such, the finding of clonal heterogeneity at diagnosis argues in favor of adopting combination rather than single-agent sequential therapies with the goal to eradicate dominant as well as minor clones that may emerge at relapse. The barcoding approach that we advocate in the current study has been shown to be very powerful in delineating the behavior of normal hematopoietic stem cells [19, 24], and will also be of benefit to the study of preclinical leukemic clonality models. Finally, we advocate to implement novel methodology in the clinic that allows to monitor the extent of leukemic clonality during disease progression and after treatment.

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SUPPLEMENTARY FIGURES

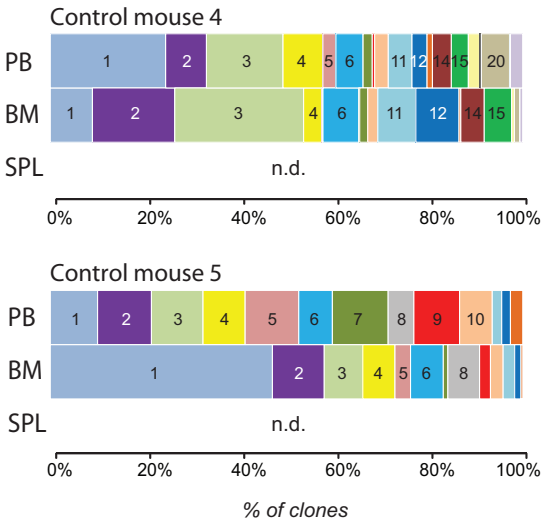

Figure S1. Clonality in control transplanted mice. Control mice were transplanted with barcoded control vector transduced cells. The number of clones and their relative contribution to blood, bone marrow and spleen was analyzed. Different clones in individual mice are indicated by different colors and their corresponding number. Note that the clone numbering and coloring relates only to clones found in an individual mouse; similarly colored and numbered clones in separate mice refer to distinct clones.

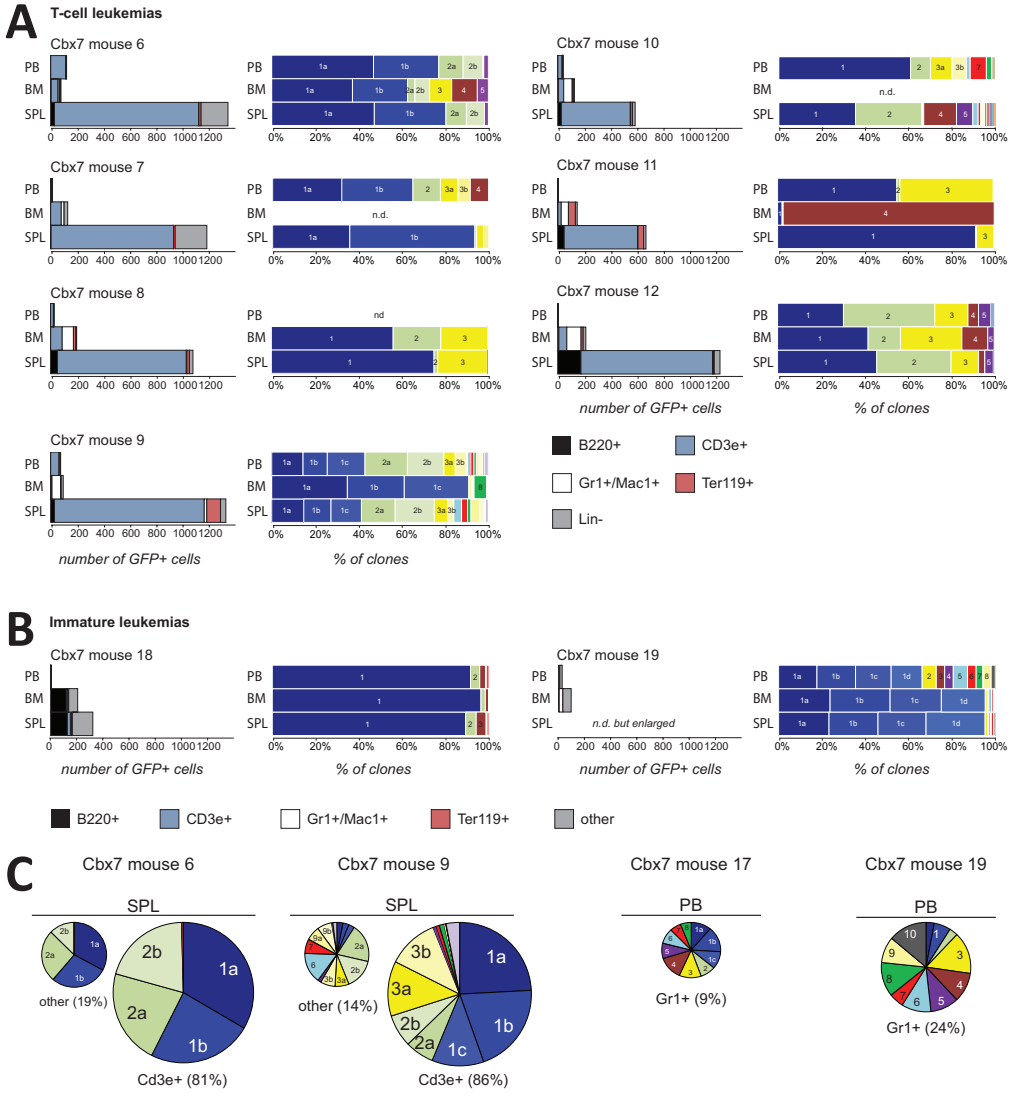

Figure S2. Clonality in Cbx7-induced leukemic mice. Ppercentage of different donor-derived GFP+ hematopoietic cell types in blood, bone marrow and spleen (left), and the contribution of different clones to blood, bone marrow and spleen (right) at the day of sacrifice at clear signs of morbidity, in **A** T-cell leukemic mice and in **B** in immature leukemias. **C-D** Different clones in different hematopoietic lineages from the spleen from T-cell leukemic mice **C** and from blood from mice with an immature leukemia (D). Different major and minor (as illustrated by the size of the pie) hematopoietic cell populations (Gr1+ granulocytes, CD3ε^{+} T-cell, and other (Lin-)) were FACS-purified on basis of marker expression, gDNA was isolated and barcodes were extracted. Similar but also different barcodes contribute to different lineages. The size of the pie illustrates the percentage of cells in the indicated tissueDifferent clones in individual mice are indicated by different colors and their corresponding number. In all mice, the barcode that was most frequently found among all samples from one transplantation experiment was numbered barcode 1 (blue), the second most frequently appearing barcode was numbered barcode 2 (green) etc. Note that the clone numbering and coloring relates only to clones found in an individual mouse; similarly colored and numbered clones in separate mice refer to distinct clones.

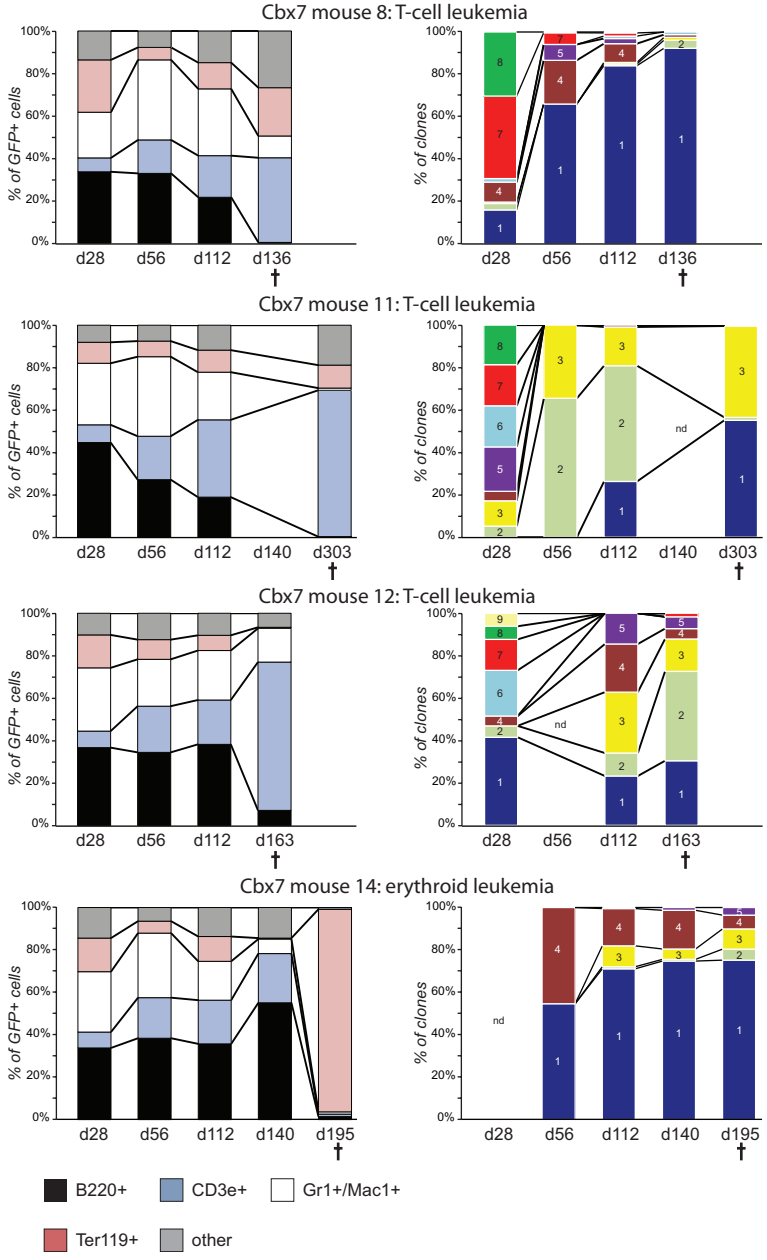

Figure S3. Onset of clonal dominance in Cbx7-induced leukemic mice. Left: percentage of different donor-derived GFP+ hematopoietic cell types in blood on indicated timepoints, as analysed by FACS. Right: The contribution of different clones to the blood on indicated timepoints. The final timepoint is the day of sacrifice at clear signs of morbidity. Different clones in individual mice are indicated by different colors and their corresponding number. In all mice, the barcode that was most frequently found among all samples from one transplantation experiment was numbered barcode 1 (blue), the second most frequently appearing barcode was numbered barcode 2 (green) etc. Note that the clone numbering and coloring relates only to clones found in an individual mouse; similarly colored and numbered clones in separate mice refer to distinct clones.

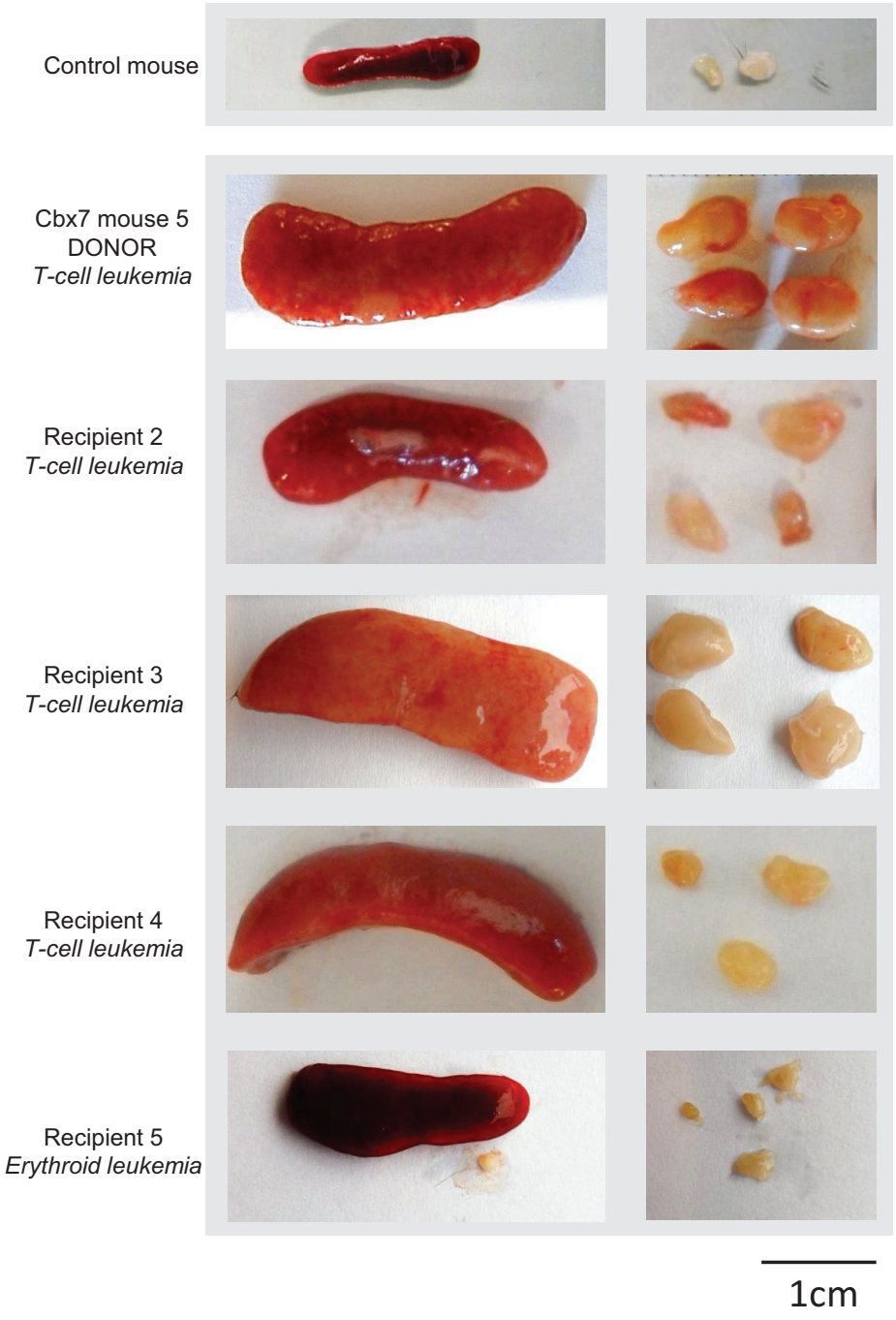

Figure S4. Appearance of a phentypically distinct leukemia after serial transplantation. Pictures of spleen and lymph nodes are depicted from a control mouse and leukemic mice from figure 7b. After serial transplantation of bone marrow cells from a mouse with a T-cell leukemia, recipient mice developed either a T-cell leukemia, or an erythroid leukemia (recipient 5).

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Chapter 6

Summary and Future Perspectives

SUMMARY

Adult stem cells that reside within different organs and tissues are critical actors for the maintenance of tissue structure and function, also referred to as 'tissue homeostasis'. By their potential to undergo self-renewal divisions, as well as to differentiate into the mature cells of their surrounding tissue, they can sustain homeostasis throughout life by replacing aged cells and repairing damaged tissue. For example, blood cells in the circulation have a limited lifespan, and are continuously replenished by hematopoietic stem cells (HSCs) that reside in the bone marrow. Although self-renewal of HSCs is essential to maintain the HSC pool throughout life, excessive self-renewal is a key characteristic of leukemic cells. Therefore, insight into the process that balances self-renewal and differentiation will provide relevant information on cellular and organismal aging, as well as on cancer (leukemia) development. Epigenetic mechanisms help to maintain the characteristic gene expression profile of stem cells, or to drive changes in gene expression that accompany the transition from hematopoietic stem cells to terminally differentiated blood cells. In true self-renewal divisions, daughter cells have to inherit the same epigenetic status as the parental cell from which they derive, to maintain a unique HSC cell signature. Thus, for a cell division to be truly self-renewing, besides accurate duplication of genomic DNA, the chromatin structure also needs to be copied and transmitted to the daughter cells. In contrast, during a differentiation division, chromatin modifications need not to be faithfully copied, leading to differential gene expression profiles and a distinct (i.e. non-stem cell) identity of daughter cells.

In this thesis, our overall aim was to further our fundamental understanding of the epigenetic machinery that distinguishes hematopoietic stem cell self-renewal divisions from differentiation divisions.

In **Chapter 1**, as an introduction, we describe a specific class of epigenetic modifiers, known as the Polycomb Group (PcG) proteins. PcGs can either maintain or modify the chromatin structure by maintaining or establishing specific histone modifications, such as H3K27me3 and H2AK119Ub. According to the 'histone code' these modifications result in compaction of chromatin and inhibition of transcription by blocking the movement of RNA Polymerase along the DNA. PcGs reside within two main multimeric complexes termed Polycomb Repressive Complex 1 and 2. Genes encoding for Polycomb group proteins have expanded during evolution, resulting in a variety of PRC1 and PRC2 subcomplexes in mammals. The effects of single PcGs by either downregulating or upregulating their expression, has revealed the importance of PcGs such as Ezh2 and Bmi1 in hematopoietic stem cells. It is generally believed that Polycomb proteins repress the transcription of lineage-specific genes in HSCs. In response to extrinsic or intrinsic signals, PcG proteins can be displaced from these genes

and be recruited to stem cell-specific self-renewal genes, to promote differentiation and suppress unlimited proliferation. However, due to the evolutionary diversification of PcG genes and proteins, a wide array of combinatorially distinct PRCs exist. To date, the role of different family members has remained unknown. Are PRC subcomplexes cell type specific? Do PRC subcomplexes have different enzymatic activities and molecular functions? Do PRC subcomplexes target different sets of genes for histone modifications?

Accumulating evidence indeed indicates that the activity of PRC1 and PRC2 is highly dependent on its exact molecular composition. In addition, data suggests that family members such as Ezh1 and Ezh2, and Bmi1 and Mel18 have distinct functions in hematopoietic cells. We therefore hypothesize that the diversity of PRC subcomplexes may regulate the dynamic equilibrium between HSC self-renewal and differentiation. Compositional rearrangements, either due to misexpression of PcG genes or due to mutations in PcG genes, may constitute oncogenic events by improper PRC enzymatic activity and target selectivity. This may result in various types of hematological malignancies, which have been reviewed in detail in this chapter.

In **Chapter 2**, we review the role of Polycomb group proteins in cellular aging and cellular transformation, and postulate that these can actually be viewed as two sides of the same coin. We speculate that PcG proteins are important in regulating the balance between aging (limited stem cell self-renewal) and the risk of developing cancer (excessive self-renewal). Both hematopoietic stem cell aging and development of hematopoietic malignancies are accompanied by the perturbance of a variety of cellular processes, including senescence, DNA repair and cell fate decisions (self-renewal vs. differentiation). While impaired self-renewal activity, skewing of myeloid differentiation, and derepression of the 'senescence' INK4a-ARF locus, are common hallmarks of normal HSC aging, malignant transformation of HSCs is generally associated with the acquisition of excessive self-renewal properties. Because of the unique self-renewal characteristics of stem cells, these cells are traditionally thought to be immortal and exempt from aging. However, this would render them very vulnerable to malignant transformation due to the potential accumulation of genetic or epigenetic lesions. The induction of HSC senescence provides a potential protective mechanism, explaining why the self-renewal potential of HSCs declines with age. The balance between self-renewal and senescence must therefore be carefully controlled. We postulate that PcG proteins are key players in this process.

As already suggested in Chapter 1, evolutionary diversification of Polycomb-encoding genes argues for the existence of cell-type- and differentiation-stage-specific PRC1 and PRC2 subcomplexes with unique molecular functions. In **Chapter 3** we studied the role of different PRC1-associated Cbx family members in HSC regulation. We show that Cbx family members

have distinct cell stage-specific expression patterns, which allows for the formation of variant PRC1 complexes during hematopoietic differentiation. As Cbx7 is highest expressed in the most immature hematopoietic populations, the PRC1 complex in HSCs preferentially contains Cbx7. By overexpressing individual Cbx genes in hematopoietic stem and progenitor cells, we show that Cbx7-containing PRC1 complexes can induce self-renewal of multipotent hematopoietic cells by repressing the expression of progenitor-specific genes. Due to the excessive self-renewal of Cbx7-overexpressed cells, mice transplanted with Cbx7-overexpressing hematopoietic stem and progenitor cells all develop leukemias. Cbx7 can be outcompeted by other Cbx7 family members, resulting in Cbx2-, Cbx4-, or Cbx8-containing PRC1 complexes. We show that these complexes inhibit HSC self-renewal by inducing entrance into the differentiation pathway by repressing HSC-specific genes. Our data support the notion that in HSCs PRC1 complexes composed of different Cbx subunits regulate the balance between self-renewal and differentiation.

In **Chapter 4** we used the Cbx7 mouse model, developed in Chapter 3, to study the behavior of leukemic stem cells (LSCs) *in vivo*. Our current understanding of the dynamics of tumor development and heterogeneity is still very limited, although this is highly relevant for existing and future therapeutic interventions. We used a previously developed barcoding tool, in which cells are marked by the introduction of a unique, heritable DNA 'barcode' in the retroviral vector. After transduction and transplantation, offspring of individual cells (clones), can be traced by sequencing and quantifying the abundance of unique barcodes. In Chapter 3, we showed that Cbx7 overexpression resulted in three different types of leukemia; immature, erythroid and T-cell leukemias. By implementation of this barcoding tool, we have generated a mouse model in which Cbx7 served as the initial leukemic 'hit' and every pre-LSCs is uniquely labelled. This approach allowed for the identification of LSC-derived clones in the transplanted primary and secondary recipients.

Although all cells of a tumor were thought to be clonally related, our model documents the coexistence of (sometimes quiescent) LSC clones with different leukemic properties in one tumor. LSC clones can aggressively compete, resulting in monoclonal leukemias, or collaborate (or at least being equally competitive) resulting in oligoclonal leukemias. Within (oligoclonal) leukemias, LSC clones can have different properties resulting in different disease phenotypes at relapse, illustrated in our model by serial transplantation studies. Quiescent LSCs can become activated resulting in phenotypically different disease at relapse. In addition, some LSC clones retain multilineage differentiation capacities and one LSC clone can therefore induce phenotypically distinct leukemias at relapse.

FUTURE PERSPECTIVES

Polycomb Cbx family members in hematopoietic stem cells

Our data, as well as previous reports [1-5], show that PRC1 complexes can be constitutionally distinct and functionally more complex than previously anticipated. Cbx proteins are mutually exclusive for PRC1, and one report showed that they specifically assemble in PRC1 complexes containing either Bmi1 or Mel18, but not in PRC1 complexes containing other Pcgf orthologs [2]. Like Cbx proteins, Bmi1 and Mel18 also show non-redundant functions in HSCs. While Bmi1 is required for long-term HSC self-renewal [6-7], Mel18 has negative effects on self-renewal [8-9]. Biochemically, Bmi1 and Mel18 also have different properties. Whereas Bmi1 is able to stimulate the E3 ubiquitin ligase activity of Ring1b, Mel18 does not exert this activity [10]. We show that, in a myeloid celline, Cbx7 and Cbx8 can assemble in both Bmi1 and Mel18-containing PRC1 complexes. Thus, Cbx7-Mel18, Cbx7-Bmi1, Cbx8-Mel18, Cbx8-Bmi1 complexes might all coexist in HSCs, but whether they have different molecular and biological functions remains to be determined. In addition, Reinberg *et al*. showed that non-canonical PRC1 complexes also exist [2] (reviewed in Chapter 1). These complexes lack Cbx, but instead contain either Rybp or Yaf2 and can bind PcG target sites independently of PRC2 and the H3K27me3 mark [11]. In embryonic stem cells, Cbx7-containing and Rybp-containing PRC1 complexes define overlapping but also different biological functions. Both PRC1 subcomplexes target many common genes, but while RYBP-PRC1 complexes also specifically target genes involved in metabolism and the cell-cycle, Cbx7-PRC1 complexes target genes involved in lineage commitment [12]. Whether these non-canonical PRC1 complexes also exist in hematopoietic cells and whether they are required for HSC regulation is subject to further study.

We identified Cbx7 as an important hematopoietic stem cell gene. By studying genome-wide binding sites of Cbx7, we showed that Cbx7 might aid to maintain progenitor-specific genes in a repressed state, thereby preventing HSCs from entering differentiation. However, the ChIP-seq experiments were performed with heterogeneous cell populations (although enriched for stem and progenitors cells), in which either Cbx7 or Cbx8 was overexpressed. In addition, since the protein levels are very high after overexpression (+1000-fold), this might potentially result in the identification of false positive targets. To reduce this possibility, we employed a very stringent protocol of identifying specific Cbx7 and Cbx8 targets. Next to using a empty vector control as a blank dataset, the sequencing profile of the data set of the alternative Cbx ChIP-seq data was used to extract specific targets. Since both Cbx7 and Cbx8 were overexpressed, and we do not focus on common targets, this should have reduced identification of false positive targets. Ideally, however, one would not use overexpression conditions to search for targets of a specific protein, but rather do ChIP-seq using antibodies against the endogenous protein. We are currently optimizing ChIP seq experiments for low

cell numbers, which would make it possible to study binding sites of different (endogenous) Cbx-containing PRC1 complexes in different rare hematopoietic cell subsets, including long-term HSCs (approximately 20.000 cells can be isolated from one mouse). This would give insight into the dynamics of Polycomb PRC1 targets during normal HSC differentiation.

It has been reported previously that Cbx7, but also other PcG proteins, can recruit DNA methyltransferases (Dnmt) to chromatin [13]. DNA methylation might thus induce another dimension of gene repression, specifically for loci bound by Cbx7-PRC1 complexes. Dnmt3a is one of the de novo methyltransferases that can actively initiate DNA methylation of unmethylated DNA, which will generally result in gene repression. Different isoforms are known to occur for Dnmt3a. Preliminary data in our lab showed that overexpression of Cbx, but not Cbx8, resulted in enhanced expression of the longer isoform (≈130 kD) of Dnmt3a, but repression of the shorter isoform (≈78 kD). Expression of the longer isoform of Dnmt3a is also found to be specific for primitive hematopoietic subpopulations, defined by L-S+K+. These results implicate a possible unique function of the long Dnmt3a isoform in HSCs. It might be of interest to study whether Cbx7 requires the long Dnmt3a isoform for stable repression of its targets in HSC, to induce HSC self-renewal.

In chapter 1, we described that due to the evolutionary diversification of PcG genes and proteins, a wide array of combinatorially distinct PRCs exist. This diversification of PRC complexes might account for tissue-specific stem cell functioning. Whereas we show that Cbx7 is the most important Cbx family member in HSCs, in epidermal stem cells Cbx4 showed to be most important [14]. However, this role seems not to be PRC1-dependent. Cbx4 exerts SUMOylation activity independent of its chromodomain to suppress terminal differentiation of epidermal cells. Instead, we show that Cbx7 requires an intact chromodomain and integration into PRC1 to induce HSC self-renewal. It remains to be determined which of the Cbx orthologs are required for other types of adult stem cells.

Cbx7 and Cbx8 knock-out mice

Tan *et al*. showed that Cbx8 deficient mice display no apparent hematopoietic phenotype [15]. The number of hematopoietic stem cells and progenitors in the bone marrow is normal, and they are functionally equivalent to their wildtype counterparts in reconstitution of the hematopoietic compartment after transplantation into irradiated recipients. However, the PRC1 complex composition in *Cbx8*$^{-/-}$ hematopoietic stem cells has not been studied. According to our results, Cbx2 and/or Cbx4 might be functionally redundant to Cbx8. In that case, Cbx2 and Cbx4 might substitute for Cbx8 and incorporate into PRC1 complexes, without any functional consequences.

Forzati *et al*. generated conventional Cbx7 KO mice [16]. According to our findings, these

mice would have reduced number of hematopoietic stem cells and potentially suffer from bone marrow failure. This has not been reported. However, during early development, full conventional knock-out mice might develop unexpected compensatory mechanisms which might obscure the study of a particular gene. Cbx7 is shown to be essential for ESCs [4-5], which makes it surprising that *Cbx7-null* mice are viable. This suggests that such compensatory effects to rescue the adverse effects of loss of Cbx7 during development might indeed have occurred. Conditional Cbx7 knock-out mice have been generated in the RIKEN Reseach Institute (dr. Koseki, RIKEN, Japan), and it might be worth studying HSCs of these mice, for example in a transplantation setting.

While no obvious hematological phenotype has been reported in adult *Cbx7-null* mice, these mice do developed liver and lung adenomas and carcinomas. The authors suggest that Cbx7 might have tumor suppressive functions by inhibiting cell cycle progression. This is supported by several other reports that showed that loss of CBX7 expression correlates with a poor prognosis in several types of cancer, such as thyroid cancer, pancreatic cancer and colon carcinoma [17-21]. On the contrary, our data suggest that Cbx7 functions as an oncogene, since its expression causes several types of lethal leukemias in mice and its protein level was found to be elevated in human lymphoid tumors [22]. This apparent discrepancy between tumor suppressive and oncogenic functions of Cbx7 might be explained by tissue-specific functions of Cbx-containing PRC1 complexes, as argued before. As discussed in Chapter 1, depending on the cell context and interacting partners, individual PcG genes may display either tumor suppressor or oncogenic functions, even within the same tissue. For example, loss or gain of Ezh2 activity (either by altered expression or by mutations) can both contribute to leukemogenesis in patients [23-24].

Extrapolation to the clinic

Regenerative medicine is expected to transition from a research promise to clinical reality in the upcoming years. However, the use of regenerative medicine relies on a proper understanding of pathways involved in stem cell specification. Modulation of the expression of genes that induce differentiation or self-renewal of HSCs by gene-therapy may be required to fully exploit the use of stem cells in future regenerative therapies. However, the introduction of new genetic material is undesired in clinical protocols since it is still under debate whether insertional mutagenesis from the vectors used to transfer genetic material can trigger clonal expansion of HSCs itself [25-26]. Therefore, small molecules that target key genes or pathways, including those found in this study, might be of use in ex vivo manipulation and perturbation of the function of stem cells. We are currently planning to explore the design of small molecules that could potentially interfer with PRC1 complex compositions. We now have several *in vitro* but also *in vivo* platforms to screen the effect of such small molecules. Potent small molecules that can 'remove' Cbx7 from the complex

are predicted to induce terminal differentiation of leukemic cells, and might therefore cure Cbx7-induced leukemias in mice. If so, molecules that interfer with PRC1 complexes might be used in a clinical trial setting. Such a strategy has been successful in the past for bromodomain-containing proteins [27].

However, the above-mentioned strategy will take multiple years to develop. In the meantime, it might be of interest to investigate the prognostic value of our findings in clinical settings. The expression of, and mutations in some Polycomb genes, is already used as a prognosis factor. We expect that Polycomb complex composition might be a more relevant predictive factor. A first step would be to screen Polycomb complex compositions in samples from patients that suffered from leukemia, such as acute myeloid leukemia, acute lymphoid leukemias and chronic myeloid and lymphoid leukemias.

Using our barcoded Cbx7-induced leukemia mouse model, we show that the clonal make-up of leukemias can be more complex than previously anticipated. We provide evidence that different LSC clones have different leukemic properties, which can become prevalent at leukemia relapse. Secondary epigenetic or genetic mutations can potentially drive tumor heterogeneity and clonal progression after relapse. It would be of interest to combine our barcoding approach to define LSC clones, together with genome sequencing to precisely identify the occurence of secondary hits. Using this method, potential tumor 'driver' mutations could be identified with higher resolution and precision than with genome sequencing alone. Our finding of leukemic LSC heterogeneity might explain why patients with relapsed leukemias who are treated similarly as when the leukemia first occurred, might show a different response to therapy [28-30]. Second, it might explain why relapsed leukemias might display different phenotypes, which has been reported recurrently [31-45]. We argue that therapies should focus on eliminating all clones within a tumor, including quiescent LSCs. As such, the finding of clonal heterogeneity at diagnosis argues in favor of adopting combination, rather than single-agent therapies with the goal to eradicate dominant as well as minor clones that may emerge at relapse.

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Appendices

DUTCH SUMMARY / NEDERLANDSE SAMENVATTING (VOOR NIET-INGEWIJDEN)

Introductie

We zijn allemaal ontstaan uit één enkele cel, gevormd door de samensmelting van de zaadcel van onze vader en de eicel van onze moeder. We erven dan ook de helft van ons genetisch materiaal (DNA) van de vader en de helft van de moeder. In het DNA liggen onze erfelijke eigenschappen verborgen, in zogenaamde genen (kleine stukjes DNA). Genen in het DNA (+/- 30.000 in de mens) bevatten de code voor de opbouw van eiwitten, de werkpaarden van een cel die specifieke functies uitoefenen. Sommige eiwitten bepalen bijvoorbeeld de kleur van onze ogen, terwijl andere eiwitten zorgen voor het omzetten van suiker in energie. In principe heeft Iedere cel in ons lichaam hetzelfde DNA en dus dezelfde genetische informatie. Echter, als we nu naar onszelf kijken zien we dat ons lichaam bestaat uit verschillende organen en weefsels die weer zijn opgebouwd uit verschillende celtypes die er allemaal heel verschillend uitzien. Bijvoorbeeld, huidcellen beschermen ons zodat schadelijke bacteriën en virussen ons lichaam niet kunnen binnendringen, terwijl levercellen enzymen aanmaken zodat we ons voedsel kunnen verteren en daar energie uit kunnen opnemen. Maar hoe kan het dat dezelfde genetische informatie leidt tot deze verschillen? Alle cellen bezitten hetzelfde DNA, en dit codeert immers voor dezelfde eiwitten. Dit komt omdat, afhankelijk van het celtype, bepaalde genen in de cel 'uit' staan, en andere 'aan'. Een voorbeeld is hemoglobine. In principe draagt iedere cel het gen voor hemoglobine, maar alleen in de rode bloedcellen staat deze ook daadwerkelijk 'aan' en wordt dit eiwit gemaakt dat zorgt voor zuurstofbinding. Niet alle eiwitten worden dus in iedere cel gemaakt. Het proces dat hiervoor zorgt heet 'epigenetica'. Epigenetische mechanismes bepalen hoe het DNA is opgevouwen in de cel, en welke stukken DNA, met daarin genen, bloot komen te liggen en kunnen worden gebruikt om eiwitten te maken, en welke daarentegen worden verborgen. De manier waarop het DNA is gevouwen bepaalt dus welke genetische informatie in het DNA wordt gebruikt voor de functie van de cel.

Zoals hierboven uitgelegd bestaan verschillende organen uit verschillende celtypes. Maar het is nog complexer. Ook ieder orgaan zelf bevat verschillende celtypes, die individueel allemaal een specifieke taak hebben maar tezamen de functie van het orgaan uitoefenen. In bijna ieder orgaan zijn stamcellen ontdekt. Stamcellen hebben de unieke eigenschap om, relatief ongelimiteerd, zichzelf te vernieuwen, waardoor vanuit één stamcel twee stamcellen ontstaan. Hierdoor blijven er gedurende ons hele leven stamcellen behouden. Tevens kunnen stamcellen uitrijpen tot alle verschillende celtypes waaruit het orgaan bestaat. Dit is noodzakelijk omdat rijpe cellen in een orgaan maar een kort leven beschoren is. Ze moeten continu vervangen worden om de functie van het orgaan te behouden . Bloedstamcellen zijn de best bestudeerde stamcellen. Deze stamcellen zitten in het beenmerg, omdat hier het bloed wordt aangemaakt. Naast dat ze zichzelf kunnen vernieuwen, kunnen ze uitrijpen

tot allerlei verschillende typen bloedcellen, zoals witte bloedcellen, rode bloedcellen en bloedplaatjes.

Ook bij deze stamceldelingen zijn epigenetische processen belangrijk. Tijdens een deling van een stamcel waarbij een nieuwe stamcel wordt gevormd moet de functie van de cel behouden blijven. Dit betekent dus concreet dat naast het erfelijke materiaal -het DNA- ook de manier waarop het DNA verpakt zit in de cel moet worden gekopieerd. Daarentegen, wanneer een stamcel moet uitrijpen tot bijvoorbeeld een rode bloedcel, zijn weer andere eiwitten in de cel nodig om de functie van de rode bloedcel te vervullen. Dit betekent dat de manier waarop het DNA zit verpakt in de cel moet veranderen. Hierdoor verandert het pakket aan eiwitten waardoor de rode bloedcel zijn specifieke taak kan uitoefenen: zuurstof transport.

Eiwitschakelaar bepaalt of stamcel zichzelf vernieuwt of gaat specialiseren
In dit proefschrift zijn Polycomb eiwitten bestudeerd. Polycomb eiwitten kunnen de wijze waarop het DNA is gevouwen in de cel veranderen. Ze bepalen dus mede welke genetische informatie in het DNA wordt gebruikt voor de functie van de cel. Polycomb eiwitten zijn belangrijk voor bloedstamcel delingen, wat in dit proefschrift is aangetoond. Ze kunnen bepalen of een stamcel zichzelf vernieuwt of dat het zal uitrijpen tot een bloedcel. Dit mechanisme is vrij ingewikkeld want verschillende Polycomb eiwitten functioneren tezamen in een groot eiwitcomplex. Voor iedere positie in dit complex concurreren verschillende Polycomb eiwitten met elkaar.

In dit proefschrift is aangetoond dat de samenstelling van dit eiwitcomplex werkt als een schakelaar die bepaalt of stamcellen uitrijpen tot rijpe bloedcellen met een specifieke functie in het lichaam, of zorgen voor de productie van nieuwe stamcellen. Het eiwitcomplex varieert in samenstelling doordat vier verschillende eiwitten al dan niet aanwezig zijn. Een van de vier eiwitten, Cbx7, moet aanwezig zijn om stamcelvernieuwing te bevorderen. Als Cbx7 ontbreekt, en één van de andere drie familieleden (Cbx2, 4 of 8) in het complex zit, wordt de aanmaak van nieuwe stamcellen geremd en specialiseren de cellen juist tot uitgerijpte bloedcellen.

Door beenmergtransplantaties uit te voeren in muizen heeft dit onderzoek ook aangetoond dat als er teveel van het Cbx7 eiwit in de stamcel aanwezig is, leukemie ontstaat. De verhouding tussen verschillende eiwitcomplexen is dan dermate verstoord dat er teveel vernieuwing van stamcellen plaatsvindt. Hierdoor ontstaat ongeremde en ongecontroleerde celgroei; kanker. Leukemie is dan ook bloedkanker die zich kenmerkt door ongecontroleerde celdelingen en een blokkade in uitrijping van stamcellen. Dus, zorgt de schakelaar voor te veel vernieuwing van stamcellen dan ontstaat leukemie, maar als cellen teveel specialiseren

kan ook bloedceltekort ontstaan, denk aan bloedarmoede. Tijdens veroudering neemt de incidentie van dit soort ziektes toe, omdat 'oude' stamcellen minder goed gereguleerd zijn. Dit komt door opstapeling van foutjes in het DNA of door fouten in de regulatie van epigenetische processen o.a. door Polycomb eiwitten. Dit onderzoek geeft daarmee 'fundamenteel inzicht in de rol van stamcellen in de subtiele balans tussen ziekte en gezondheid' (persbericht UMCG, 28 maart 2013).

Er zijn in de afgelopen jaren veel studies gedaan om kennis te vergaren over het mechanisme van stamcel delingen. De studie in dit proefschrift heeft daar aan bijgedragen. Het is echter nog een grote stap om de bevindingen te vertalen naar de kliniek. Hypothetisch gezien zou het veranderen van de samenstelling van het Polycomb eiwitcomplex in het laboratorium kunnen leiden tot een vermeerdering van stamcellen voor een beenmergtransplantatie. Hoe meer stamcellen kunnen worden getransplanteerd, hoe beter dit is voor de patiënt. Eén methode om dit te doen is door bepaalde stofjes die aangrijpen op dit eiwitcomplex toe te voegen aan het medium waarin de cellen groeien. Een andere methode is om stamcellen genetisch te manipuleren door het inbrengen van extra stukjes DNA van genen die coderen voor eiwitten in het complex. Echter, niet van alle manipulatiestrategieën die in het laboratorium worden toegepast zijn de lange termijn effecten bekend en daarom wordt het nog niet veilig bevonden voor toepassing in de kliniek. Er is dus nog een lange weg te gaan voordat nieuw bevonden kennis en toepassingen in het laboratorium van nut zijn voor patiënten. Een goede samenwerking tussen wetenschappers in het laboratorium en artsen in de kliniek is dus essentieel en zal uiteindelijk resulteren in een verbeterde zorg voor de patiënt.

Niet alle cellen in een kanker zijn hetzelfde

Dit onderzoek heeft ook bijgedragen aan de kennis van het ontstaan en de ontwikkeling van leukemie in het bloedvormend systeem. Hiervoor is een zogeheten 'DNA barcode' gebruikt. In dit onderzoek zijn extra kopieën van het Cbx7 gen in het DNA van muizenstamcellen gebracht, maar daarnaast ook een uniek variabel stukje DNA, een 'barcode'. Iedere individuele stamcel draagt dus een unieke 'barcode'. Deze barcode wordt bij iedere celdeling gekopieerd en geërfd door de dochtercellen. Hierdoor is het mogelijk om te zien welke bloedcellen door de stamcel worden geproduceerd. Een groep cellen die wordt gevormd uit één stamcel heet een 'kloon'. Alle cellen van dezelfde kloon hebben dus dezelfde 'barcode'.

Stamcellen kunnen, nadat ze een foutje in de DNA code hebben opgelopen, of een fout in de manier waarop het DNA verpakt zit in de cel, ongeremde en ongecontroleerde groei laten zien en klonen vormen die bijdragen aan leukemie ontwikkeling. Deze studie laat zien dat een leukemie niet altijd bestaat uit cellen die allemaal identiek zijn. De leukemie kan bestaan uit meerdere klonen en dit maakt het gedrag van de leukemie complex.

Verschillende leukemieklonen kunnen verschillende eigenschappen hebben met betrekking tot groei en uitrijping (type leukemie), en verschillend reageren op stress (zoals bijvoorbeeld chemotherapie). In deze studie is dit aangetoond door leukemiecellen van een muis met een leukemie (donor), door te transplanteren in andere ontvangermuizen. In sommige ontvangermuizen resulteerde dit in een andere uitrijping van de cellen en verandering in het type leukemie (bijvoorbeeld rode bloedcel leukemie), in vergelijking tot het type leukemie dat oorspronkelijk aanwezig was in de donor muis (bijvoorbeeld witte bloedcel leukemie).

Als we deze bevindingen vertalen naar de kliniek betekent het dat leukemie bij mensen complexer kan zijn dan eerder werd gedacht. Als er meerdere klonen aanwezig zijn in een patiënt, kunnen deze verschillend reageren op chemotherapie. Deze studie laat zien dat sommige klonen heel klein kunnen zijn en blijven 'slapen' in het beenmerg na stress. Bij een patiënt kan dit betekenen dat deze kloon niet reageert op de chemobehandeling, terwijl andere klonen wel worden uitgeroeid. Deze 'slapende' leukemie klonen kunnen mogelijk jaren later leiden tot een tweede leukemie met andere eigenschappen. Aangezien leukemie dus kan bestaan uit meerdere klonen met verschillende eigenschappen, benadrukt deze studie het nut en de mogelijke noodzaak van combinatie-therapie bij de behandeling van leukemie. Hierbij worden verschillende medicijnen tegelijk gebruikt. Dit heeft als doel het uitroeien van zichtbare maar ook onzichtbare 'slapende' klonen die anders jaren later nog terugval kunnen veroorzaken.

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Een lab kan niet zonder goede analisten, ze zorgen voor het dagelijks reilen-en-zeilen en hebben veel (vaak meer dan AIO's!) ervaring met lab-technieken. Gerald, je hebt een geweldig analisten-team samengesteld, dat mede verantwoordelijk is voor de successen in het lab.

Ellen, zonder jouw gouden handen hadden we nooit genoeg experimenten bij elkaar kunnen pipetteren voor de NCB publicatie. Samen met jou experimenten doen heb ik altijd erg gezellig gevonden. Ik vind je eerlijkheid heel prettig, daardoor weet ik altijd precies waar ik aan toe ben. We werken nu voor het zesde jaar samen, en ik hoop dat deze samenwerking de komende jaren net zo prettig zal zijn. **Mathilde**, wat een geluk dat jij in mijn derde jaar kwam als nieuwe analist in ons lab (bedankt voor het ' koppelen' Sandra O!). Ik heb je natuurlijk niet voor niets gevraagd om mijn paranimf te zijn (nee, je hoeft geen rokje aan!). Je bent een harde werker en net als Ellen, heb jij gouden handen. Inmiddels ben je zo ongeveer onmisbaar geworden voor het lab, wat soms misschien wel wat zwaar op je schouders drukt. Maar zie het als compliment. Naast dat je een collega bent, zie ik je toch ook wel als vriendin. De avonturen die we samen hebben meegemaakt zal ik met een lach op mijn gezicht blijven herinneren. Denk aan je autocross wedstrijd, ons bezoekje aan meneer Hulzebosch, het stappen in Kopenhagen, ons snelweg-avontuur, etc... Er zullen vast nog meer avonturen volgen! **Martha R.**, met jou en Ronald deelde ik het kantoor op de vijfde en we hebben veel gezellige(oa over UFO's) en ook persoonlijke gesprekken gehad. Jij hebt me altijd geholpen met alle muizen-experimenten, en ik ben je daar heel dankbaar voor. Het is niet altijd prettig wat we de diertjes aan moeten doen, maar je deed het altijd met zoveel mogelijk liefde en zachtaardigheid. Dit is heel bewonderenswaardig. Je bent en blijft een grote dierenvriend. **Bertien**, hetzelfde geldt eigenlijk voor jou. Jij bent ook geweldig in het doen van *in vivo* experimenten, maar je regelt ook alle organisatie daaromheen. Ik ben blij dat je dit ons allen uit handen neemt. Ook in het lab ruim je vaak zonder gemopper de rommel van anderen op, en vul je de voorraad bij. Je bent zo snel. Ik wil graag mijn

waardering daarvoor uitspreken. Daarnaast ben je een hele fijne collega, waar ik altijd op kan bouwen. **Erik**, als enige kerel tussen al die vrouwen heb je het vast niet altijd gemakkelijk ;) Of misschien vind je het stiekem toch eigenlijk wel leuk. Ik word vaak al duizelig als ik naar jouw computerscherm kijk, bio-informatica blijft voor mij abacadabra. Ik ben blij dat jij dat een leuk vak vindt, en dat je hiermee ons lab bent komen versterken. We hebben samen, en met Lenja, grote datasets weten te veranderen in begrijpelijke figuren en conclusies. Ik kom ook graag even bij je bureau langs om een praatje maken, met een kopje koffie erbij. Dus, bedankt voor je hulp en gezelligheid!

En dan zijn er nog drie analisten die inmiddels niet meer op de stamcel afdeling werkzaam zijn maar die ik toch graag zou willen bedanken. **Bert**, jij ging al bijna met pensioen toen ik net als AIO begon. Ik heb van jou nog geleerd hoe je CFU-GMs moest inzetten en scoren en ik heb als eerst met jou meegekeken hoe je beenmergcellen moet isoleren. Bedankt! Ik hoop dat je volop geniet van je vrijheid en pensioen! **Sandra O**, we hebben te kort mogen genieten van jouw harde werken. Jij kwam ons lab versterken toen ik net in mijn tweede jaar (?) zat. Je hebt me heel veel geholpen met cobblestones scoren (uiteraard!), maar ook met alle analyses die gedaan moesten worden op de cellen van de zieke muizen. Bedankt! We hebben ook veel lol gehad, en zelfs nog een korte tijd samen gesport (fitness en squash). En toen ging je verhuizen, terug naar je 'roots'. Ik vind het leuk dat we af en toe nog contact hebben. Jaring, je kwam als uitmuntende student van Brad bij ons op het lab, en daarna heb je ongeveer een jaar bij ons als analist gewerkt. Je hebt ons allemaal veel geleerd van FACS, bedankt! Je protocollen worden nog steeds veelvuldig gebruikt, evenals de *Jaring-assay*.

And of course, I would like to thank all my current colleague PhD students in the stem cell biology department. **Evgenia**, I enjoyed working with you and discussing about scientific but also non-scientific topics. Our trip with Maarten, Alice and Brad to a 'sunny' desert island in the Whitsundays of Australia was quite an experience and this will stay a living memory for me (with good pictures!). You are about to finish, so I wish you all the best in this final phase of your PhD. **Višnja**, we have worked a lot together on your 'Dnmt project' and my 'Cbx project' and we also wrote a review together. I appreciate all your help and efforts in the lab. I hope to see your thesis soon! **Edyta**, you are relatively new in the lab, but you already have generated a large set of data. You are a very hard worker, and I am confident that you will finish your PhD successfully. **Sara**, you smart girl. Still a long way to go before you will finish your PhD, but time will fly! We started a project together, and I sincerely hope that it will result in a nice paper. I also wish you all the best with the *histone-content-during-aging project*. This was your own idea which you brought into the lab when you started. Also many thanks for your moral support which you and Sophia gave me during the stressful revision period of the NCB paper.

Other PhD students already finished and left the stem cell biology department to start a new path in their career. **Marta**, we started our PhD about the same period. We did not work much together, but I remember hearing in my office when you were having a good laugh in the lab on the 5th floor while doing experiments. Having fun doing science is important :). Thank you! **Alice**, we hebben redelijk veel samengewerkt tijdens onze AIO periode, en we zetten dat nu voort in een andere setting (de MPN Stichting). Ik vind het leuk om je af en toe nog te spreken en te zien. We hebben allebei veel meegemaakt, en dat verbindt toch wel. **Sandra R**. we shared the office for a short period of time. You were about the finish your PhD when I started, and I remember that you were working day and night on your computer to make the deadlines of your papers. Thanks for the chats and laughs we had. **Leonie**, we hebben eigenlijk nooit samengewerkt, maar je hebt zoveel sporen nagelaten in het lab dat het eigenlijk toch wel zo voelt. We hebben elkaar redelijk goed leren kennen tijdens congressen en daar veel plezier gehad. Sommige collega's noemen mij de 'tweede Leonie'. Jij en ik zijn er samen nog niet uit of dat nu positief of negatief is bedoeld, of wel? Bedankt voor al de tips die je mij hebt gegeven gedurende mijn PhD (je weet waar ik het over heb…;)

During my PhD, I also had the honor to guide several Bachelor, Master and Topmaster students during their internships. You have all contributed to the research in this thesis, thank you. I enjoyed supervising you very much, it gave me much pleasure in the day-to-day lab-life. Thanks to (in order of appearance): **Hedy** van Wijngaarden, **Anne-Bart** Seinen, **Femke** Feringa, **Karina** Wakker, **Reinier** Bron, **Klaas** van der Laan, **Violeta** Stojanovska and **Marta** Vilà González. I hope I have contributed, in some extent, to your professional development, and I wish you all good luck in your (scientific) carreers!

Graag zou ik ook het secretariaat en het ERIBA-management willen bedankt voor hun support. Allereerst **Annet**, toen we nog op de vijfde verdieping zaten stond je altijd klaar om te helpen met financiële en organisatorische zaken. Bedankt! Ik ben blij dat je bent mee verhuisd naar het ERIBA. **Sylvia** en **Maria** zijn in het ERIBA het team komen versterken, en ik heb ook al mogen kennis maken met hun vriendelijkheid en behulpzaamheid. Bedankt! Ook zou ik graag de receptionisten van het ERIBA, **Joke** en **Kim** willen bedanken. Het is altijd prettig om een vriendelijk gezicht te zien en een kort praatje te maken als je het gebouw binnenloopt om aan je werkdag te beginnen. Ook wil ik graag het management bedanken. Op de vijfde verdieping was dat **Gerrie Hoogenberg** en in het ERIBA **Henk Heidekamp** en **Helena Rico**.

Our lab also collaborates intensively with other labs, for example with the Experimental Hematology Department headed by prof. **E. Vellenga** and prof. **J.J. Schuringa**. I enjoyed the early Monday-morning meetings, and learned a lot from the more clinical-oriented science in your presentations. So thanks to all members of this department. I would like

to thank some members of this department personally. **Jan-Jacob** en **Edo**, bedankt voor jullie kritische blikken en vragen, deze waren heel verhelderend. **Bart-Jan**, ook bedankt voor je kritische vragen en hulp bij sommige moleculair biologische vragen in mijn beginjaren. **Vincent**, je hebt nog een tijdje bij de SCB gewerkt. Ik herinner me jou vooral in je blauwe trui in de koude kamer :). Alweer een aantal jaren geleden ben je verhuisd naar de hematologie. Bedankt voor de discussie die we hadden.

I would also like to thank all **previous and current members from the departments from prof. Kampinga, prof. Sibon and prof.** Coppes of the fifth floor. Een paar mensen zou ik graag in het bijzonder willen bedanken. **Ody**, ik ben als student van de celbiologie cursus als eerste bij jouw afdeling op de vijfde verdieping terecht gekomen. Ik had toen samen met Nynke (nu post-doc bij Rob, het wereldje is klein..) het geluk om door **Willy** wegwijs gemaakt te worden op het lab. Wat een energie! Ik heb veel geleerd, en ook veel gelachen. Willy en Ody, bedankt voor deze eerste kennismaking met de wetenschap. **Harrie**, ik ben vervolgens stage gaan lopen bij jou en **Michel**. Ik heb een ontzettend leuke en leerzame tijd bij jullie op het lab gehad. Tijdens mijn stage op de afdeling heb ik veel leuke momenten gehad en uitstapjes met o.a. **Floris**, **Maria**, **Michel**, **Jurre** en **Erwin**, bedankt! Mede door deze positieve ervaring ben ik, dan wel bij een andere afdeling, weer op de vijfde terug gekomen als AIO bij Gerald. Het contact tussen mij en de mensen in jullie labs is altijd goed en gezellig gebleven. **Jeannette**, **Bart**, **Hette** en **Maria**, ik ken jullie nu al zoveel jaren, bedankt voor al jullie behulpzaamheid en gezelligheid. **Marianne** Z, naast een vriendin en een vriendin-van-vrienden, ben jij toch ook enkele jaren mijn collega-aan-de-andere-kant-van-de- gang geweest. Bedankt voor de gezellige koffie pauzes en kletsmomentjes in de gang. Most members of the department of R. Coppes I still see quite often during our Thursday meetings, but we have also shared the lab for a few years. Thanks for the collaboration and fun, **Rob**, **Marianne** van der Zwaag, **Miriam**, **Yamini**, **Martti** and **Sarah**, and from the radiotherapy 'division' **Ghazale** and **Sonja**.

In the new ERIBA building, we now share the lab and open office with the members from the departments from prof. **Lansdorp** and dr. **Foyer**. I would also thank all these lab members for the nice atmosphere at work. **Sandra H**, bedankt voor onze gezellige koffiepauzes die zijn begonnen op Schiermonnikoog! **Niek** en **Evert-Jan**, jullie hebben nog een tijd bij ons op de vijfde op het lab gewerkt, bedankt voor de gezelligheid! **Nancy**, bedankt voor je gezelligheid. **Petra**, ik heb als derdejaars studente nog een paar weken bij jou een practicum gedaan ('jullie studenten kunnen echt nog helemaal niets!"). Leuk om jou weer zoveel te zien!

I also had the opportunity to work as a guest-researcher for a short period of time during my PhD in the department of **Kristian Helin**, in Copenhagen, Denmark. There I learned all in's and out's (well, as much as you can learn in a few months) about immune-precipitation from Xudong Wu, a very experienced post-doc. **Xudong**, you are a great teacher with a lot of

patience! In just a few weeks I did so many experiments, it resulted in about two full figures of the NCB paper. This would have never been possible without your help and guidance in the lab, but also your scientific view was indispensable. I am really happy we are still in contact! **Susanne**, bedankt voor je gezelligheid en gastvrijheid tijdens mijn bezoek aan jullie lab. Je stond altijd klaar om vragen die ik had over de technieken te beantwoorden, of je hielp me met zoeken als ik weer eens iets niet kon vinden. Ik heb de etentjes bij jou en Ingmar thuis erg gezellig gevonden. **Kristian**, our short meetings during my stay were very valuable to me. I appreciate that you welcomed me in your lab, and I learned a lot.

Ik heb ook veel uren doorgebracht in het cytometrie-centrum. Ik heb altijd veel leuke gesprekken gehad tijdens het sorteren met **Geert**, **Henk**, **Roelof-Jan** en in het verleden ook **Harold**. Ook ontzettend bedankt voor alle keren dat jullie langer bleven omdat ik toch nog een paar minuutjes langer wilde door sorteren. Dat getuigt van betrokkenheid, en het wordt erg gewaardeerd! Geert, jouw kennis van FACS is echt onuitputtelijk. Mede hierdoor is hoogstaand onderzoek waarbij FACS komt kijken mogelijk. Roelof-Jan, bedankt voor die avond dat we, op speciaal verzoek, in de avonduren tot wel middernacht hebben gesorteerd, met pizza nootjes en (geen) bier! Henk, ik zal de volgende keer mijn zweepje proberen thuis te laten ;) alhoewel het erop lijkt dat je mijn figuurlijke slagen stiekem toch wel kan waarderen.

Alhoewel niet allemaal succesvol, heb ik ook nog veel experimenten gedaan in samenwerking met de Genetica afdeling. **Pieter van der Vlies**, **Bahram** en **Jelkje**, bedankt voor jullie hulp en geduld! Ook wil ik graag alle mensen van het CDP bedanken, voor alle goede zorgen voor mijn muizen.

En last-but-not-least, zou ik graag mijn familie en vrienden willen bedanken. Allereerst mijn lieve vriendinnetjes **Seda** en **Patrizia**. Seda, je zit momenteel veeeel te ver weg. Je bent ontzettend reislustig waardoor ik je de laatste tijd wel erg veel moet missen. We zijn vaak na het werk wat gaan eten, en wezen stappen. Hierdoor kon ik me weer opladen voor het werk. Graag had ik de traditie om ieder jaar samen een lang weekend weg te gaan naar een leuke stad in ere gehouden, maar dat gaat nu natuurlijk even niet lukken. Je hebt vaak een andere kijk op dingen dan de meeste mensen, en daarom ben je ook zo belangrijk voor mij. BEDANKT! Patrizia, ik heb niet voor niets jou gevraagd om mijn paranimf te zijn. Samen met Richard heb je me altijd in alles ontzettend gesteund. Het wetenschaps-wereldje kennen jullie helemaal niet, maar jullie zijn altijd vol belangstelling. Ik vind het altijd erg fijn om na een lange dag hard werken bij jullie gezin (met de liefste kleine meisjes!!!) bij de eettafel te kunnen aanschuiven. Als ik bij jullie ben, voel ik me thuis, en kan ik alles (ook het werk) even vergeten. BEDANKT! Ook wil ik mijn andere goede vriendinnen bedanken voor alle gezelligheid buiten het werk, **Betty**, **Esther** en **Lisa**.

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Lieve **pap**, ik vind het heel spijtig dat je de grote ceremoniële dag niet mee kan maken. Je was altijd vol interesse, en kwam vaak met allerlei (kranten)artikelen aanzetten over stamcellen en hematologie. Maar mijn promotietraject duurde lang, te lang voor jou. Mijn onderzoek heeft raakvlakken met de ziekte die jij hebt gehad, en daardoor zijn sommige aspecten van mijn werk soms erg confronterend, maar toch ook mooi. Ik ben met de neus op de feiten gedrukt van het belang van onderzoek over bloed stamcellen en leukemie. Door wat we hebben meegemaakt zet ik me nu ook vrijwillig in voor andere patiënten door mijn werk voor de MPN Stichting. Bedankt voor alle fijne jaren, en alle steun en hulp die ik van je heb gekregen. Ik hoop dat je kleine 'puk' je een beetje trots heeft gemaakt.

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CURRICULUM VITAE

Personal Details

Name	Karin Klauke
Date and place of birth	18-01-1983, Groningen, the Netherlands

Professional Experience

2013-present	Post-doctoral investigator at the Laboratory of Ageing Biology and Stem Cells (Prof. dr. G. de Haan), European Research Institute for the Biology of Ageing, University Medical Centre Groningen, University of Groningen, Groningen, the Netherlands
2007-2013	PhD student at the Laboratory of Ageing Biology and Stem Cells (Prof. dr. G. de Haan), European Research Institute for the Biology of Ageing, University Medical Centre Groningen, University of Groningen, Groningen, the Netherlands
2011	Guest researcher Laboratory of prof. K. Helin, Biotech Research & Innovation Centre, University of Copenhagen. Feb-Apr 2011
2006/2004	Teaching assistant practical classes “Oncology” second-year students Life-Science and Technology, University of Groningen. 1 month
2005	Teaching assistant tutorial classes “Statistics” first-year Biology students, University of Groningen. 1 month

Boards and committees

2013-present	Board member of the European MPN Advocates Network; a collaborative network of existing MPN foundations across Europe
2012-present	Board member of the dutch MPN foundation
2004-2005	WINGS-ESN, Erasmus Student Network, committee member

Education

2007-2013	PhD in Medical Sciences, University of Groningen, the Netherlands
2001-2007	B.Sc. and M.Sc. (cum laude) in Medical Biology, University of Groningen, the Netherlands

Courses

International

2010	1st workshop in Chromatin Proteomics, Ludwig Maximilians University of Munich, Germany
2009	Hydra V, the European Summer School on Stem Cells and Regenerative Medicine, organized by EuroSystems, Hydra, Greece

National

2008-2011	Project Management, Graduate School GUIDE, University of Groningen
2010	Publishing in English, Graduate School GUIDE, University of Groningen
2008	Article 9, Laboratory Animal Sciences, University of Groningen
2008	Epigenetics in Health and Disease, Boerhaave course, University of Leiden
2008	Technical & Ethical aspects of Digital Image Manipulation, Graduate School GUIDE, University of Groningen

Awards

New Investigator Award, International Society of Experimental Hematology (ISEH)
2013, Vienna, Austria

International Society of Experimental Hematology (ISEH), travel award based on abstract ranking
2012, Amsterdam, the Netherlands
2011, Vancouver, Canada
2010, Melbourne, Australia
2009, Athens, Greece

International Society for Stem Cell Research (ISSCR), travel award based on abstract ranking
2012, Yokohama, Japan

Finalist young investigator award, International Society of Experimental Hematology (ISEH)
2010, Melbourne, Australia

Travel Award, 1st workshop in Chromatin Proteomics, Ludwig-Maximilians University
2010, Munich, Germany

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